Remarks

I. Status of the Claims

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 94-95, 99-103, 105-107, and 131-146 are pending in the application, with claims 94, 107, and 139 being the independent claims. The Examiner has indicated that claims 94, 95, 99-103, 105, and 106 are allowable. Claims 108, 111, 112, 115, 116, 118-124, 126, 127, and 130 are canceled without prejudice to or disclaimer of the subject matter therein. Applicant reserves the right to pursue the canceled subject matter in related cases. Claim 107 has been amended. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicant respectfully requests that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

II. Support for Amendments

Support for the amendment to the claims can be found throughout the original claims and the specification. In particular, support for claims 107 and 131-146 can be found at page 43, line 5 to page 53, line 3 and page 64, line 22 to page 67, line 21.

III. Statement of Substance of the Interview

Applicant thanks Examiner Ford and Supervisory Examiner Minnifield for the courtesy extended in the Interview of October 18, 2007 with Applicant's representative,

Elizabeth J. Haanes. The rejections regarding SEQ ID NO:2 as well as the art of record were discussed. The undersigned agreed to present arguments demonstrating written description support and non-obviousness of the claim amendments presented herein.

IV. Rejection Under 35 U.S.C. §112, First Paragraph Is Mooted.

The Examiner has rejected claims 107, 108, 111, 112, 115, 116, 118-124, 126, 127, and 130 for alleged lack of enablement. The Examiner interpreted the pending claims to read on fragments and variants, which according to the Examiner, were outside the scope of enablement provided by the specification.

Solely to expedite the prosecution of this application and not in acquiescence of the Examiner's rejection, Applicant has amended claim 107, canceled claims 108-130 and submitted new claims 131-146. Claim 107 has been amended to read on a vaccine comprising an isolated polypeptide which is required to include the entire mature pmpE polypeptide. New claim 139 reads on a vaccine comprising an isolated polypeptide which is required to include the entire pmpE polypeptide encoded by the *Chlamydia* insert in the deposited plasmid M15pREP (pQE-pmpE Ct)#37. Other than the naturally-occurring mature form of pmpE which is produced by SEQ ID NO:2, claim 107 does not read on smaller fragments or variants. Accordingly, Applicant respectfully requests that this rejection be reconsidered, and further that it be withdrawn.

During the interview held on October 18, 2007, Applicant's representative agreed to make arguments demonstrating written description support for the proposed amendment to claim 107. Claim 107 is directed to a vaccine comprising an isolated polypeptide which comprises mature pmpE. Given the overwhelming understanding of secreted proteins and the

structure of secretory signal peptides, a person of ordinary skill in the art would clearly understand that Applicant was in possession of mature pmpE for use in a vaccine at the time of filing of the present application, even though the specification does not give specific amino acid coordinates of the mature protein. Accordingly, claim 107 meets the written description requirement of 35 U.S.C. § 112, first paragraph.

The test for the written description requirement is whether one skilled in the art can reasonably conclude that the inventor has possession of the claimed invention in the specification as filed. *Vas-Cath Inc. v. Mahurkar,* 935 F.2d 1555, 1563, 19 U.S.P.Q.2d 1111, 1116 (Fed. Cir. 1991); M.P.E.P. § 2163.02. The Federal Circuit recently re-emphasized the well-settled principle of law that "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [they] invented what is claimed,'" *Union Oil of Cal. v. Atlantic Richfield Co.,* 208 F.3d 989, 54 U.S.P.Q.2d 1227 (Fed. Cir. 2000).

The Examiner's attention is further drawn to *Falkner v. Inglis* 448 F.3d 1357 (Fed. Cir. 2006). In *Falkner*, the interference count was to a recombinant poxvirus in which an essential gene had been inactivated. Inglis's specification spoke generally of deleting essential genes from viruses, and provided detailed examples of deletion of essential genes from an unrelated virus, herpesvirus. While use of poxviruses in the invention was contemplated, not a single recombinant poxvirus with a deleted essential gene was described. Indeed not a single example of a poxvirus gene, whether essential or not, was described. *Id.* at 1364. Testimony showed that essential genes in poxviruses were known in the art, noting that "the skilled person would have been readily able to choose an essential vaccinia gene."

Id. at 1366. Based on this testimony, and in spite of the fact that not a single essential poxvirus gene was disclosed, let alone inactivating such a gene in a recombinant virus, the Federal Circuit held that:

> in accordance with our prior case law, that (1) examples are not necessary to support the adequacy of a written description (2) the written description standard may be met (as it is here) even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure.

Id. Thus, the court held that Inglis's disclosure, which did not describe a single essential gene of a poxvirus nonetheless provided adequate written description for a recombinant poxvirus with an inactivated essential gene. Quoting *Capon v. Eschar* (418 F.3d 1349, 76 USPQ2d1078 (Fed. Cir. 2005)), the *Falkner* court noted that the "text needed to meet [the written description requirement] varies with the nature and scope of the invention at issue, and *with the scientific and technologic knowledge already in existence.* *Id.* at 1367 (emphasis added, internal citations omitted).

A broad base of scientific and technical knowledge relating to secreted proteins has been in place for at least 25 years. *See e.g.*, Lewin, B., *Genes*, John Wiley and Sons (1983), at pp. 159-160 (attached hereto as Exhibit 1). Even in 1983 it was well understood that "the N-terminus of secreted proteins consists of a cleavable leader of from 16-29 amino acids, which starts with two or three polar residues, but continues with a high content of hydrophobic amino acids . . . ." *Id.* at 159. As is well known in the art, once a signal peptide is cleaved, what is left is the "mature" polypeptide. The knowledge since 1983 has become increasingly more sophisticated such that signal peptides can be easily predicted based on the amino acid structure. Indeed, an internet-based algorithm for predicting signal peptides of secreted proteins was available in 1997. *See, e.g.*, Nielsen *et al. Protein Engineering 10*:1-6

(1997) at page 5 (attached hereto as Exhibit 2). The most recent version of this program may be found at www.cbs.dtu.dk/services/SignalP/ (visited October 30, 2007).

As these references clearly demonstrate, the structure of a secretory signal peptide, and thus the structure of the mature protein remaining upon cleavage of the signal peptide, was well known and would have been easily predictable from a polypeptide sequence. As such, a person of ordinary skill in the art would have immediately understood that Applicant was in possession of the mature form of pmpE for use in a vaccine. Under such circumstances, as stated by the Federal Circuit in *Falkner*, "there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure." *Falkner* at 1366. Accordingly, Applicant respectfully asserts that the specification provides more than adequate written description support for claim 107 as amended.

V. The Claims are Novel

Claims 107, 111-112, 115-116, 118-124, and 130 are rejected under 35 U.S.C. § 102(b) over Griffais *et al.* (International Publication No. WO 99/28475, published June 10, 1999) ("Griffais"), under 35 U.S.C. §102(a) over Probst *et al.* (International Publication No. WO 00/34483, published June 15, 2000) ("Probst PCT"), and under 35 U.S.C. §102(e) over Probst *et al.* (U.S. Patent No. 6,432,916, filed April 19, 2000 and issued August 13, 2002) ("Probst"). *See* Office Action at pages 7-8; *See also* Non Final Office Action mailed on August 20, 2006 at page 15.

Applicant respectfully reminds the Examiner that Probst PCT was cited by the Examiner in an earlier non-final Office Action mailed on June 01, 2001. In response,

Applicant submitted a Declaration Under 37 C.F.R. §1.131 by Dr. W. James Jackson to antedate the conception of the present invention to a date prior to June 15, 2000. The Examiner, in response, noted that "[t]he Declaration and attached exhibits 1-12-C filed on December 2, 2001 under 37 CFR 1.131 are sufficient to overcome the Probst et al (WO 00/34483, published June 15, 2000) reference." Office Action mailed May 14, 2002 at page 2. Therefore, as acknowledged by the Examiner, Probst PCT is not available as a reference. With respect to the other cited references as applied to the amended claims, Applicant respectfully traverses.

The Examiner noted that "claims 107-108 and 118 - 124 [sic: 14], 126-127, and 130 recite 'an amino acid sequence of SEQ ID NO: 2,'" and thus the Examiner considered that the rejected claims are directed to fragments of SEQ ID NO: 2. Office Action at page 10.

Solely to expedite the prosecution and not in acquiescence of the Examiner's rejection, Applicant has amended claim 107, deleted claims 108-130, and submitted new claims 131-146. Claim 107 has been amended to read on a vaccine comprising an isolated polypeptide which is required to include the entire mature pmpE polypeptide. New claim 139 reads on a vaccine comprising an isolated polypeptide which is required to include the entire pmpE polypeptide encoded by the *Chlamydia* insert in the deposited plasmid M15pREP (pQE-pmpE Ct)#37. Other than the naturally-occurring mature form of pmpE which is produced by SEQ ID NO:2, claim 107 does not read on smaller fragments or variants.

The Examiner also noted that "claims 111- 112, 115- 116 and dependent claims (118-124, 126-127 and 130) are product -by-process claims which have no *structural attributes*." Office Action at page 10. (Emphasis in original). It is respectfully submitted that the

currently pending claims, while containing a functional limitation, do not include product by process limitations.

In view of the amendments and arguments, Applicant respectfully requests that the rejection be reconsidered and withdrawn.

VI. The Currently Pending Claims are Non-Obvious

Claims 107, 108, 111, 112, 115, 116, 118-124, 126, 127, and 130 are rejected under 35 U.S.C. § 103(c) over Probst and in further view of Murdin *et al.* (*Infection and Immunity*, October 1993, p. 4406-4414). *See* Office Action at pages 14 and 15; *See also* Non-Final Office Action mailed on August 30, 2005, at pages 14-15. In particular, the Examiner stated that the rejected claims are considered to encompass the fragments of SEQ ID NO: 2 for the same reason that the claims recite the terms "an amino acid sequence of SEQ ID NO: 2." *See* Office Action at page 15.

Solely to facilitate prosecution of this application and not in acquiescence of the Examiner's rejection, Applicant has amended claim 107, deleted claims 108-130, and submitted new claims 131-146. Claim 107 has been amended to read on a vaccine comprising an isolated polypeptide which is required to include the entire mature pmpE polypeptide. New claim 139 reads on a vaccine comprising an isolated polypeptide which is required to include the entire pmpE polypeptide encoded by the *Chlamydia* insert in the deposited plasmid M15pREP (pQE-pmpE Ct)#37. Other than the naturally-occurring mature form of pmpE which is produced by SEQ ID NO:2, claim 107 does not read on smaller fragments or variants. Insofar as the rejection applies to the new claims, however, Applicant respectfully disagrees.

The United States Supreme Court recently addressed the issue of obviousness in *KSR International Co. v. Teleflex Inc.*, 550 U.S. ___ (2007). The Court stated that the *Graham v . John Deere Co. of Kansas City*, 383 U.S. 1 (1966) factors still control an obviousness inquiry. Those factors are: 1) "the scope and content of the prior art"; 2) the "differences between the prior art and the claims"; 3) "the level of ordinary skill in the pertinent art"; and 4) objective evidence of nonobviousness (*KSR*, 127 S.Ct. at 1734 (quoting *Graham*, 383 U.S. at 17-18)).

In proceedings before the Patent and Trademark Office, the Examiner bears the burden of establishing a *prima facie* case of obviousness based upon the prior art. *See In re Piasecki,* 745 F.2d 1468, 1471-73, 223 USPQ 785, 788 (Fed. Cir. 1984). In order to establish a *prima facie* case of obviousness, the Examiner's analysis must support that the claimed invention would have been obvious to one of ordinary skill in the art after consideration of all the facts. 35 U.S.C. 103(a). The mere fact that the references can be combined or modified does not render the resultant combination obvious unless the results would have been predictable to one of ordinary skill in the art. *See* M.P.E.P. §2143 at 2100-140 (Rev. 6, Sept. 2007); *See also* the recently published "Examination Guidelines" for determining whether claims are non-obvious in view of the *KSR* holding. 72 FR 57526 (October 10, 2007).

Furthermore, Post-*KSR* decisions by the Federal Circuit clearly indicate that the requirement for showing a reasonable expectation of success still plays an important role in an obviousness determination and, further, that evidence demonstrating a lack of a reasonable expectation of success must be considered. *See, e.g., Takeda Chemical Industries, Ltd. v. Alphapharm PTY., LTD*, 492 F.3d 1350, 83 U.S.P.Q.2d 1169 (Fed. Cir. 2007); and *Forest Laboratories, Inc. v. IVAX Pharmaceuticals, Inc.*, No. 2007-1059, slip op. (Fed. Cir. Sept. 5,

2007). Although these important post-*KSR* decisions were not discussed in the "Examination Guidelines," they are instructive for applying post-*KSR* obviousness analysis.

For example, in *Takeda*, the court determined that, even though a compound similar to the claimed anti-diabetic compound, piaglitazone, was known in the prior art, one of ordinary skill would not have had a reasonable expectation of success in obtaining the claimed invention. *Takeda*, 491 F.3d at 1360-62, U.S.P.Q.2d at 1177-78. The evidence showed that the prior art compound had undesirable side effects, but only two modifications were required to make the claimed compound from the prior art compound. *Id.* at 1360, U.S.P.Q.2d at 1177. Although the methods for making these modifications (*i.e.*, "homologation" and "ring-walking") were known in the art, the district court nevertheless concluded that the evidence favored a finding that one of ordinary skill in the art would not have had a reasonable expectation that performing these modifications "would cause a compound to be more efficacious or less toxic." *Id.* at 1361, 83 U.S.P.Q.2d at 1177-78. Accordingly, the Federal Circuit affirmed the holding of non-obviousness. *Id.* at 1362-63, 83 U.S.P.Q.2d at 1177.

Similarly, in *Forest Laboratories*, the Federal Circuit upheld a district court's finding that one of ordinary skill would not have had a reasonable expectation of success in attempting to separate a substantially pure enantiomer of citalopram. *Forest Labs.*, slip op. at 5. Even though the racemic mixture of citalopram was known in the art, the district court held that there was no reasonable expectation of success because there was evidence that others had tried unsuccessfully to resolve racemic citalopram, that one of the named inventors attempted the separation only as a last resort, and that one of ordinary skill in the art would have faced "a real possibility that the resolved intermediate would re-racemize during the

attempt to convert it from the diol intermediate enantiomer to the desired citalopram enantiomer." *Id.* at 4-5, 9-11. The Federal Circuit, again, affirmed the district court's finding of non-obviousness. *Id.* at 11. Thus, where evidence showed the lack of a reasonable expectation of success in the art, there was no *prima facie* obviousness.

The Cited Art Provides No Reasonable Expectation of Success

In both *Takeda* and *Forest Laboratories*, the claimed invention was deemed non-obvious based on evidence that the skilled artisan would have perceived major obstacles preventing a reasonable expectation of success. Such is the present case.

The present claims require that the claimed vaccine be protective, as evidenced by the well-accepted mouse infertility model described in the specification at page 64, line 25 through page 65, line 24. *Chlamydia* vaccine research and development is very complicated and unpredictable. Accordingly, a person of ordinary skill in the art would have had no reason to select pmpE from the large array of *Chlamydia* proteins disclosed in Probst for use as a vaccine. Due to the unpredictability, there was no reasonable expectation of success in making a protective *Chlamydia* vaccine comprising pmpE. In addition, even assuming, *arguendo*, that a *prima facie* case of obviousness was established, it is rebutted by secondary indicia such as unexpected results and long-felt but unmet need.

In support of these contentions, Applicant provides herewith a Declaration under 37 C.F.R. § 1.132 of W. James Jackson, Ph.D. ("Jackson Declaration"), along with accompanying Exhibits A-H cited therein. Dr. Jackson, the sole inventor of the present application, is an expert in the field of infectious disease and vaccine research and development.

The U.S. Patent and Trademark Office must consider evidence provided in the form of expert declarations when making an obviousness determination. *See In re Sullivan*, No. 2006-1507 (Fed. Cir. Aug. 29, 2007). In *Sullivan*, the Federal Circuit recently vacated a decision by the Board that affirmed the Examiner's obviousness rejection of claims to an antibody composition against snake venom. *Id.* The Federal Circuit reversed, concluding that the Board did not properly consider the three expert declarations that were submitted as rebuttal evidence against the obviousness rejection. *Id.* at 8.

In the cited Probst reference, the inventors discuss a large array of *Chlamydia* polypeptides, setting all of them out as potential vaccine candidates. Indeed, Probst singles out pmpC, pmpD, pmpE, and pmpI as containing "predictable signal peptides, suggesting that they are outer membrane proteins, and therefore, potential immunological targets." Probst at col. 46, lines 48-51. While in *KSR*, the Supreme Court dusted off the "obvious to try" standard, they noted that the standard only applies where "there are a finite number of identified, predictable solutions, [such that] a person of ordinary skill has good reason to pursue the known options within his or her technical grasp." *KSR*, 550 U.S. at __, slip op. at 17. The key term here, as it is throughout the *KSR* opinion, is *predictable*.

On the other hand, *Chlamydia* vaccine research, as explained in the Jackson Declaration, is decidedly *unpredictable*. According to the Declaration:

> [a] *Chlamydia* protein may be antigenic, but may not be immunogenic; a *Chlamydia* protein may be immunogenic, but may not induce an immune response sufficient to provide protection against *Chlamydia* infection; a *Chlamydia* protein may induce a humoral immune response, but may not induce a cell-mediated immune response; and, a *Chlamydia* protein may induce a strong immune response (both humoral and cell-mediated), but may not protect an animal against *Chlamydia* infection. In addition, a given *Chlamydia* protein might produce a protective immune response against the serovar from which it was derived, but not against any of the other 18-21

> recognized *C. trachomatis* serovars known to cause infection and disease in humans.

at paragraph 18. As explained by Dr. Jackson through his discussion of several literature references, a simple determination of surface exposure or immunogenicity of a *Chlamydia* polypeptide is no indication whatsoever of whether a given polypeptide will make a suitable vaccine. Furthermore, as explained in paragraph 13 of the Jackson Declaration, of the "surface proteins" predicted by Probst, only pmpG, pmpH, and pmpE are major constituents of the outer membrane of *C. trachomatis*.

In sum, the Declaration clearly demonstrates that at the time of filing, it was not easily predictable or foreseeable to a person of ordinary skill in the art whether a *Chlamydia* vaccine comprising a given polypeptide would induce a humoral and/or a cell-mediated immune response or provide a cross-protection against a *Chlamydia* infection. Accordingly, Dr. Jackson concluded that:

> a scientist in the field of *Chlamydia* vaccine research would have had no guidance as to which of the myriad polypeptides disclosed in Probst might result in a successful, cross-protective vaccine. The scientist would have had to laboriously clone, express, and purify all of the polypeptides, and then blindly test each one in a challenge model, with no assurance that any one of the polypeptides, let alone pmpE, would work.

Jackson Declaration at paragraph 19. This is decidedly *not* the "obvious to try" standard contemplated by the Supreme Court in *KSR*. Instead the present situation is fully analagous to that noted by the Federal Circuit in *Takeda* where "the prior art disclosed a broad selection of compounds any one of which could have been selected as a lead compound for further investigation." *Takeda*. at 1359. Accordingly, as with the chemical modifications in *Takeda* the person of skill in the art, in trying to identify a protective *Chlamydia* vaccine from the large array of polypeptides disclosed in Probst, would be faced with a blank slate of

unpredictable possibilities. Murdin does not cure the deficiency, because it only provides a peptide fragment T-cell epitope of an entirely unrelated *Chlamydia* protein. In view of the unpredictability in *Chlamydia* vaccine research and development set forth above in the Jackson Declaration, the cited references fail to provide a reasonable expectation that the vaccine comprising a pmpE polypeptide would be useful as a *Chlamydia* vaccine and would, upon administration to female mice, reduce *Chlamydia trachomatis*-induced infertility as required by the claims. Thus, the cited references fail to demonstrate *prima facie* obviousness of the pending claims.

Secondary Indicia Support Non-Obviousness

Even assuming, *arguendo*, that a *prima facie* case of obviousness was established, secondary indicia of non-obviousness, namely unexpected results and unmet need, would fully rebut such a finding.

Dr. Jackson explains in detail that a desirable quality in a *Chlamydia* vaccine is the ability to protect against multiple serovars. Dr. Jackson explains that post filing data available in the CIP of the present application showed that a pmpE polypeptide derived from serovar L_2 protected against *Chlamydia trachomatis*-induced infertility upon challenge with *C. trachomatis* serovar F. Since almost all *Chlamydia* vaccine candidates which have been tested are only serovar specific (with exceptions stated in the Declaration), Dr. Jackson concludes that "the fact that the pmpE L_2 vaccine of the present invention provided protection against a serovar F challenge was surprising." Jackson Declaration at paragraph 19. Thus, the ability of pmpE to be cross-protective was unexpected.

In addition to the unexpected properties, the Declaration points out at paragraphs 19 and 20 that there was a long-felt and unmet need for the technology of the claimed invention

because of the importance of providing *Chlamydia* vaccines which are cross-protective against different *Chlamydia* serotypes. *Id.* at paragraphs 19-20. For example, the Dr. Jackson explains that, due to its unique life cycle and its ability to avoid host's immune response, *Chlamydia* infection often produces serious disease. *Id.* Furthermore, Dr. Jackson notes that even though *Chlamydia* infections are susceptible to antibiotic treatment, vaccination is imperative because *Chlamydia* infections often escape immune surveillance and diagnosis in the earlier, treatable stages of disease. *Id.* Thus, the Declaration concludes that compositions comprising the claimed isolated pmpE polypeptide fulfill a long-felt need, and overcome earlier failed vaccine compositions. *Id.*

Thus, in addition to the lack of a *prima facie* case of obviousness as discussed above, the secondary indicia of non-obviousness discussed in the Jackson Declaration further support the conclusion that the present claims are non-obvious over the cited art.

Based on this discussion, Applicant respectfully urges the Examiner to reconsider any pending obviousness rejections, as they apply to the currently pending claims.

Conclusion

All of the stated grounds of objection and rejections have been properly traversed, accommodated, or rendered moot. Applicant therefore respectfully requests that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicant believes that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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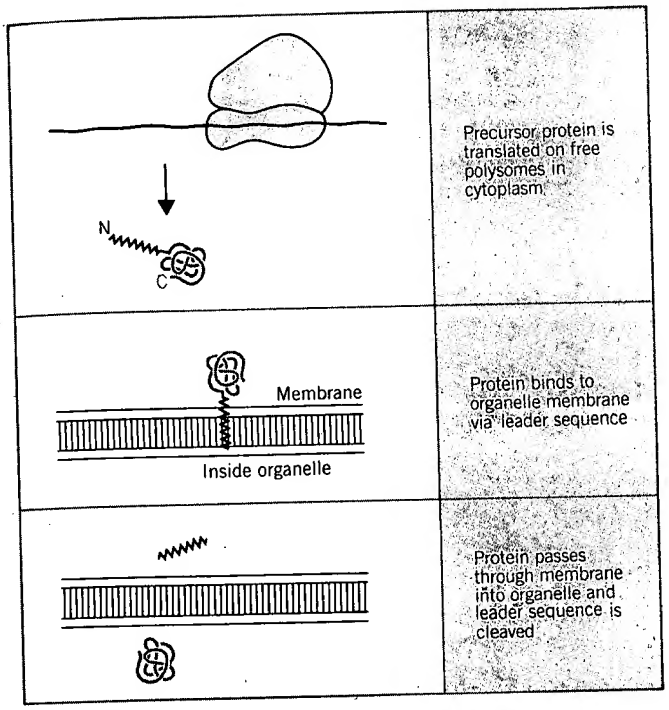


Figure 9.12
Leader sequences are used for proteins to recognize mitochondrial or chloroplast surfaces.

For many proteins that must be inserted in membranes, the sequence of the mature polypeptide is not itself sufficient to direct membrane insertion. Additional information is needed; and this most often takes the form of a **leader sequence** at the N-terminal end of the protein. The protein carrying this leader is called a **preprotein.** It is a transient precursor to the mature protein, since the leader is cleaved as part of the process of membrane insertion.

The **pre** sequence is distinct from the **pro** sequence that describes the additional regions present on proteins that exist as *stable* precursors. Some proteins may have both. For example, insulin is initially synthesized as **preproinsulin;** the **pre** sequence is cleaved during secretion, generating **proinsulin,** which is the substrate for processing to mature insulin.

The leader sequence plays different roles in different circumstances. For certain proteins synthesized within the cytoplasm, but destined to reside within the chloroplast or mitochondrion, the product of cytoplasmic protein synthesis is a precursor some 5000 daltons (roughly 45 amino acids) larger than the mature protein. This precursor is released from polysomes. If it is added to intact organelles *in vitro,* it can be incorporated into the compartment. As illustrated in **Figure 9.12,** this involves passage through the organelle membrane, during which the leader sequence is cleaved, probably by a protease located on the outside of the envelope. The leader sequence serves to provide information recognized by the organelle membrane and used to sequester the protein in a **post-translational** process. Note that a cleavable leader is not the only acceptable form of such information; some mitochondrial proteins are recognized as such in their mature form, and may have an internal sequence that is able to ensure membrane passage without cleavage.

For proteins that are secreted through, or inserted into, other cellular membranes, the process of association most often starts during translation. The polyribosomes synthesizing these proteins are associated with the membrane of the endoplasmic reticulum. The preproteins are not released into the cytoplasm to form a precursor pool, but instead pass directly from the ribosome to the membrane. From the membrane, the proteins enter the Golgi apparatus, and then are directed to their ultimate destination, such as the lysosome or the plasma membrane.

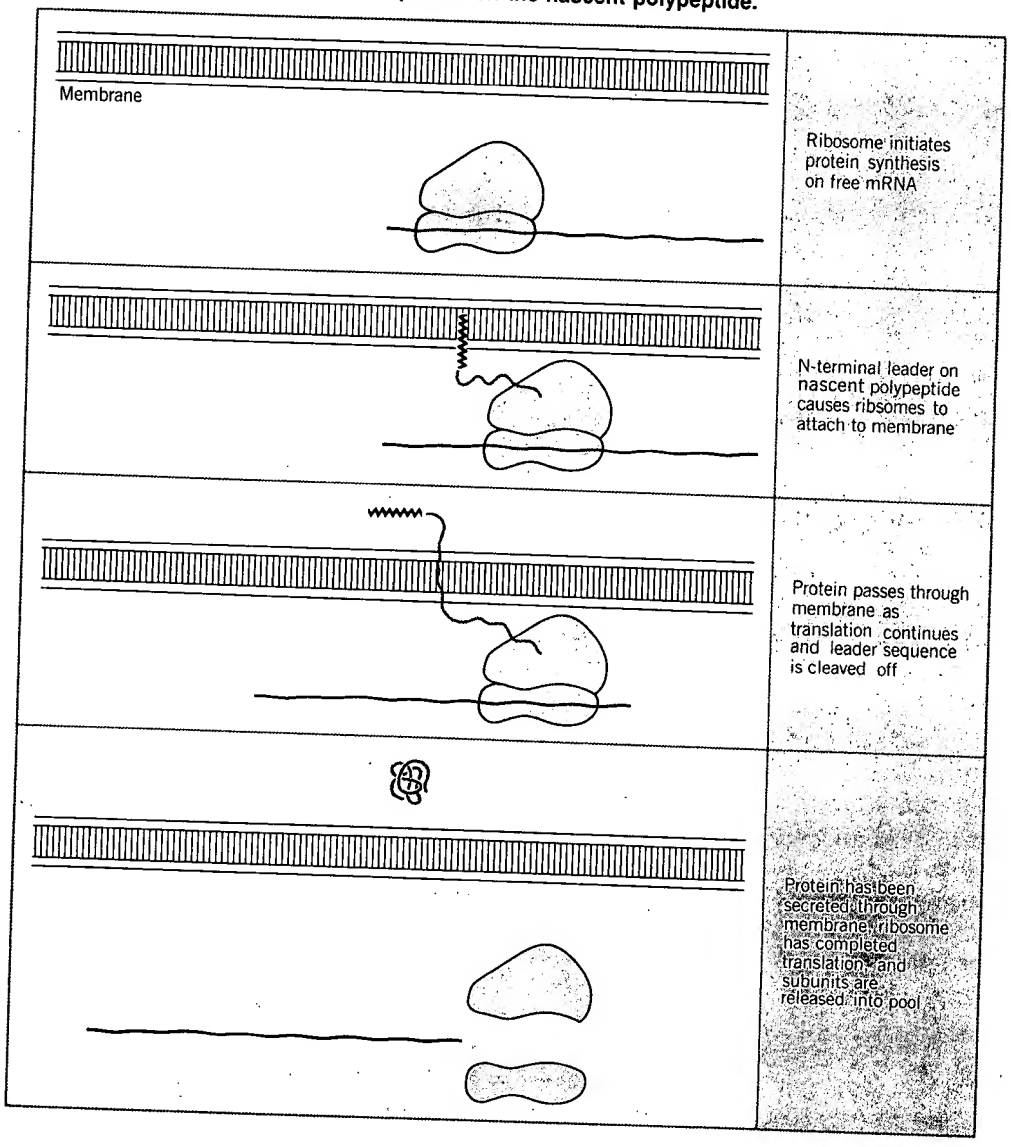
A model for the mechanism of membrane insertion has been based on work with eucaryotic microsomal systems (containing ribosomes and endoplasmic reticulum). These systems are able to package *nascent* proteins into membranes; but they do not work with the addition of isolated preproteins. The **signal hypothesis** proposes that the leader characteristic of almost all secreted proteins constitutes a **signal sequence** whose presence distinguishes them from other proteins. With only rare exceptions, the N-terminus of secreted proteins consists of a cleavable leader of from 16 to 29 amino acids, which starts with two or three polar residues, but continues with a high content of hydrophobic amino acids; otherwise there is no noticeable conservation of sequence.

The signal sequence provides the means for ribosomes translating the mRNA to attach to the membrane. Some membrane receptor recognizes the signal sequence, perhaps by virtue of its hydrophobicity,

and inserts the precursor protein directly into the membrane, probably as soon as the signal sequence and a few additional amino acids have been synthesized. A route to characterizing the protein receptors is provided by the discovery that salt-washed membranes cannot sponsor ribosomal attachment; but this ability can be recovered by adding the salt wash. The active component has been purified in the form of a complex of six proteins.

Figure 9.13 shows that as synthesis of the nascent polypeptide chain continues, there comes a point at which the protein is well inserted into the membrane, and the signal sequence can be cleaved. Then when the ribosomes complete translation, the protein is al-

Figure 9.13
The signal hypothesis proposes that ribosomes synthesizing secretory proteins are attached to the membrane via the leader sequence on the nascent polypeptide.



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SHORT COMMUNICATION

Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites

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We have developed a new method for the identification of signal peptides and their cleavage sites based on neural networks trained on separate sets of prokaryotic and eukaryotic sequence. The method performs significantly better than previous prediction schemes and can easily be applied on genome-wide data sets. Discrimination between cleaved signal peptides and uncleaved N-terminal signal-anchor sequences is also possible, though with lower precision. Predictions can be made on a publicly available WWW server.

Keywords: cleavage sites/protein sorting/secretion/signal peptide

Introduction

Signal peptides control the entry of virtually all proteins to the secretory pathway, both in eukaryotes and prokaryotes (Gierasch, 1989; von Heijne, 1990; Rapoport, 1992). They comprise the N-terminal part of the amino acid chain and are cleaved off while the protein is translocated through the membrane. The common structure of signal peptides from various proteins is commonly described as a positively charged n-region, followed by a hydrophobic h-region and a neutral but polar c-region. The (–3,–1) rule states that the residues at positions −3 and −1 (relative to the cleavage site) must be small and neutral for cleavage to occur correctly (von Heijne, 1983, 1985).

A strong interest in the automated identification of signal peptides and the prediction of their cleavage sites has been evoked not only by the huge amount of unprocessed data available, but also by the industrial need to find more effective vehicles for the production of proteins in recombinant systems. The most widely used method for predicting the location of the cleavage site is a weight matrix which was published in 1986 (von Heijne, 1986). This method is also useful for discriminating between signal peptides and non-signal peptides by using the maximum cleavage site score. The original matrices are commonly used today, even though the amount of signal peptide data available has increased since 1986 by a factor of 5–10.

Here, we present a combined neural network approach to the recognition of signal peptides and their cleavage sites, using one network to recognize the cleavage site and another network to distinguish between signal peptides and non-signal peptides. A similar combination of two pairs of networks has been used with success to predict the intron splice sites in pre-mRNA from humans and the dicotelydoneous plant *Arabidopsis thaliana* (Brunak *et al.*, 1991; S.Hebsgaard, P.Korning, J.Engelbrecht, P.Rouźe and S.Brunak, submitted). Artificial neural networks have been used for many biological sequence analysis problems (Hirst and Sternberg, 1992; Presnell and Cohen, 1993). They have also been applied to the twin problems of predicting signal peptides and their cleavage sites, but until now without leading to practically applicable prediction methods with significant improvements in performance compared with the weight matrix method (Arrigo *et al.*, 1991; Ladunga *et al.*, 1991; Schneider and Wrede, 1993).

Materials and methods

The data were taken from SWISS-PROT version 29 (Bairoch and Boeckmann, 1994). The data sets were divided into prokaryotic and eukaryotic entries and the prokaryotic data sets were further divided into Gram-positive eubacteria (*Firmicutes*) and Gram-negative eubacteria (*Gracilicutes*), excluding *Mycoplasma* and *Archaebacteria*. Viral, phage and organellar proteins were not included. In addition, two single-species data sets were selected, a human subset of the eukaryotic data and an *Escherichia coli* subset of the Gram-negative data.

The sequence of the signal peptide and the first 30 amino acids of the mature protein from the secretory protein were included in the data set. The first 70 amino acids of each sequence were used from the cytoplasmic and (for the eukaryotes) nuclear proteins. In addition, a set of eukaryotic signal anchor sequences, i.e. N-terminal parts of type II membrane proteins (von Heijne, 1988), were extracted (see Figure 1).

As an example of a large-scale application of the finished method, we used the *Haemophilus influenzae* Rd genome—the first genome of a free-living organism to be completed (Fleischmann *et al.*, 1995). We have downloaded the sequences of all the predicted coding regions in the *H.influenzae* genome from the World Wide Web (WWW) server of the Institute for Genomic Research at http://www.tigr.org/. Only the first 60 positions of each sequence were analysed.

We have attempted to avoid signal peptides where the cleavage sites are not experimentally determined, but we are not able to eliminate them completely, since many database entries simply lack information about the quality of the evidence. The details of the data selection are described in the WWW server and in an earlier paper (Nielsen *et al.*, 1996a).

Redundancy in the data sets was avoided by excluding pairs of sequences which were functionally homologous, i.e. those that had more than 17 (eukaryotes) or 21 (prokaryotes) exact matches in a local alignment (Nielsen *et al.*, 1996a). Redundant sequences were removed using an algorithm which guarantees that no pairs of homologous sequences remain in the data set (Hobohm *et al.*, 1992). This procedure removed 13–56% of the sequences. The numbers of non-homologous sequences remaining in the data sets are shown in Table I. Redundancy

Table I. Data and performance values

Source	Data					
	(Number of sequences)		Network architecture (window/hidden units)		Performance	
	Signal peptides	Non-secretory proteins	*C*-score	*S*-score	Cleavage site location (% correct)	Signal peptide discrimination (correlation)
Human	416	251	15+4/2	27 / 4	68.0 (67.9)	0.96 (0.97)
Eukaryote	1011	820	17+2/2	27 / 4	70.2	0.97
E.coli	105	119	15+2/2	39 / 0	83.7 (85.7)	0.89 (0.92)
Gram–	266	186	11+2/2	19 / 3	79.3	0.88
Gram+	141	64	21+2/0	19 / 3	67.9	0.96

Data: the number of sequences of signal peptides and non-secretory (i.e. cytoplasmic or nuclear) proteins in the data sets after redundancy reduction. The organism groups are eukaryotes, human, Gram-negative bacteria ('Gram–'), *E.coli* and Gram-positive bacteria ('Gram+'). The human data are subsets of the eukaryotic data and the *E.coli* data are subsets of the Gram-negative data. The signal anchor and *H.influenzae* data are not shown in the table. *Network architecture*: the size of the input window and the number of hidden computational units ('neurons') in the optimal neural networks chosen for each data set. *C*-score networks have asymmetrical input windows. *Performance*: the percentage of signal peptide sequences where the cleavage site was predicted to be at the correct location according to the maximal value of the Y-score (see Figure 2). The ability of the method to distinguish between the signal peptides and the N-terminals of non-secretory proteins (based on the mean value of the *S*-score in the region between position 1 and the predicted cleavage site position) is measured by the correlation coefficients (Mathews, 1975). Both performance values are measured on the test sets (the average of five cross-validation tests). The values given in parentheses indicate the performance for the human sequences when using networks trained on all eukaryotic data and for the *E.coli* sequences when using Gram-negative networks respectively.

reduction was not applied to the signal anchor data or the *H.influenzae* data, since these were not used as training data.

Neural network algorithms

The signal peptide problem was posed to the neural networks in two ways: (i) recognition of the cleavage sites against the background of all other sequence positions and (ii) classification of amino acids as belonging to the signal peptide or not. In the latter case, negative examples included both the first 70 positions of non-secretory proteins and the first 30 positions of the mature part of secretory proteins.

The neural networks were feed-forward networks with zero or one layer of two to 10 hidden units, trained using back-propagation (Rumelhart *et al.*, 1986) with a slightly modified error function. The sequence data were presented to the network using sparsely encoded moving windows (Qian and Sejnowski, 1988; Brunak *et al.*, 1991). Symmetric and asymmetric windows of a size varying from five to 39 positions were tested.

Based on the numbers of correctly and incorrectly predicted positive and negative examples, we calculated the correlation coefficient (Mathews, 1975). The correlation coefficients of both the training and test sets were monitored during training and the performance of the training cycle with the maximal test set correlation was recorded for each training run. The networks chosen for inclusion in the WWW server have been trained until this cycle only.

The test performances have been calculated by cross-validation: each data set was divided into five approximately equal-sized parts and then every network run was carried out with one part as test data and the other four parts as training data. The performance measures were then calculated as an average over the five different data set divisions.

For each of the five data sets, one signal peptide/non-signal peptide network architecture and one cleavage site/non-cleavage site network architecture was chosen on the basis of the test set correlation coefficients. We did not pick the architecture with absolutely the best performance, but instead the smallest network that could not be significantly improved by enlarging the input window or adding more hidden units.

The trained networks provide two different scores between zero and one for each position in an amino acid sequence. The output from the signal peptide/non-signal peptide networks, the *S*-score, can be interpreted as an estimate of the probability of the position belonging to the signal peptide, while the output from the cleavage site/non-cleavage site networks, the *C*-score, can be interpreted as an estimate of the probability of the position being the first in the mature protein (position +1 relative to the cleavage site).

If there are several *C*-score peaks of comparable strength, the true cleavage site may often be found by inspecting the *S*-score curve in order to see which of the *C*-score peaks coincides best with the transition from the signal peptide to the non-signal peptide region. In order to formalize this and improve the prediction, we have tried a number of linear and non-linear combinations of the raw network scores and evaluated the percentage of sequences with correctly placed cleavage sites in the five test sets. The best measure was the geometric average of the C-score and a smoothed derivative of the S-score, termed the Y-score:

$$Y_i = \sqrt{C_i \Delta_d S_i}, \tag{1}$$

where $\Delta_d S_i$ is the difference between the average *S*-score of d positions before and d position after position i:

$$\Delta_d S_i = \frac{1}{d}\left(\sum_{j=1}^{d} S_{i-j} - \sum_{j=0}^{d-1} S_{i+j} \right) \tag{2}$$

In Figure 2(A), examples of the values of the C-, S- and Y-scores are shown for a typical signal peptide with a typical cleavage site. The C-score has one sharp peak that corresponds to an abrupt change in the S-score from a high to low value. Among the real examples, the C-score may exhibit several peaks and the S-score may fluctuate. We define a cleavage site as being correctly located if the true cleavage site position corresponds to the maximal Y-score (combined score).

For a typical non-secretory position, the values of the C-, S- and Y-scores are lower, as shown in Figure 2(B). We found the best discriminator between signal peptides and non-secretory

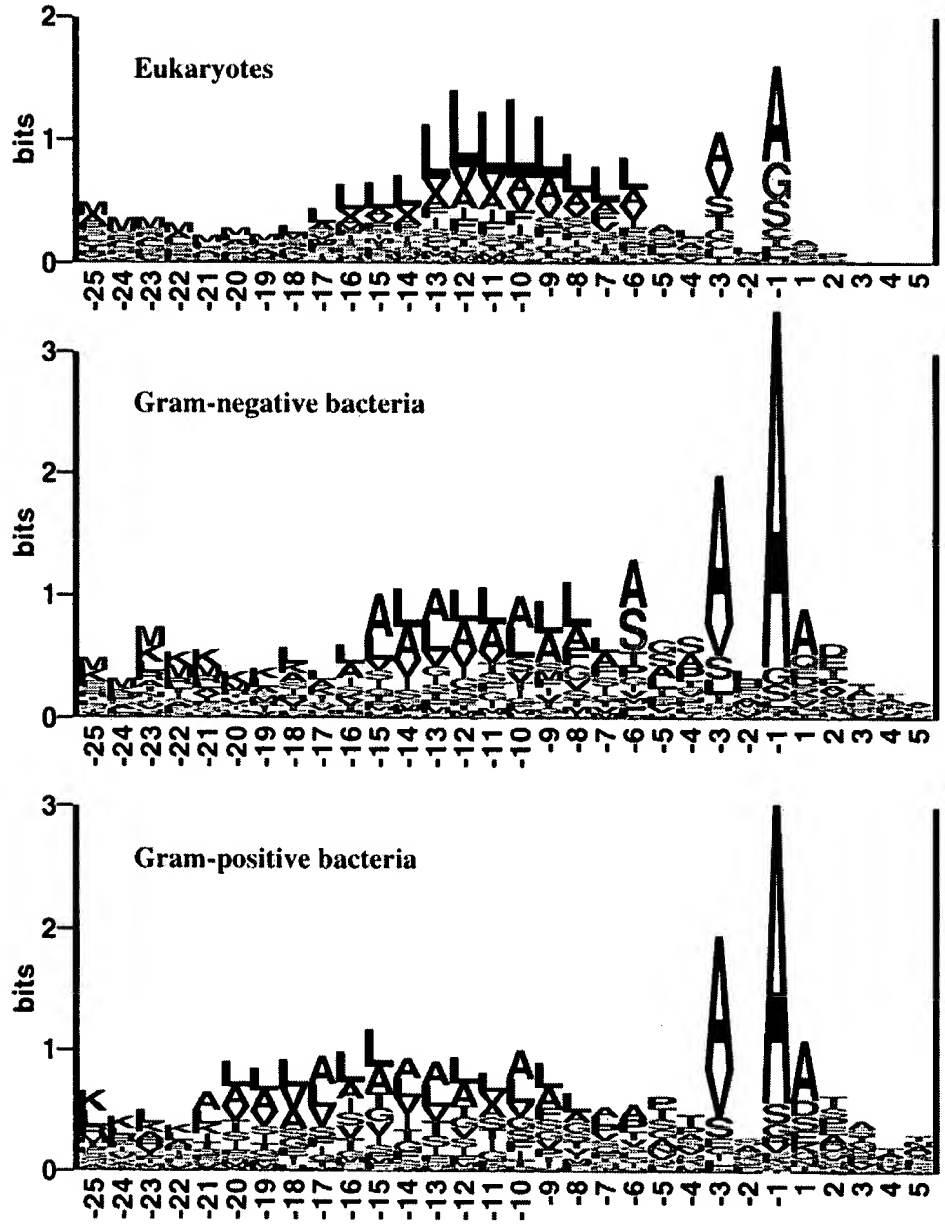

Fig. 1. Sequence logos (Schneider and Stephens, 1990) of signal peptides, aligned by their cleavage sites. The total height of the stack of letters at each position shows the amount of information, while the relative height of each letter shows the relative abundance of the corresponding amino acid. The information is defined as the difference between the maximal and actual entropy (Shannon, 1948): $I_j = H_{max} - H_j = \log_2 20 + \Sigma_\alpha\, n_j(\alpha)/N_j \log_2 n_j(\alpha)/N_j$, where $n_j(\alpha)$ is the number of occurrences of the amino acid α and N_j is the total number of letters (occupied positions) at position j. Positively and negatively charged residues are shown in blue and red respectively, while uncharged polar residues are green and hydrophobic residues are black.

proteins to be the average of the S-score in the predicted signal peptide region, i.e. from position 1 to the position immediately before the position where the Y-score has a maximal value. If this value—the mean S-score—is greater than 0.5, we predict the sequence in question to be a signal peptide (cf. Figure 3).

The relationship between the various performance measures and their development during the training process is described in detail elsewhere (Nielsen *et al.*, 1997).

Results and discussion

The optimal network architecture and corresponding predictive performance for all the data sets are shown in Table I. The C-score problem is best solved by networks with asymmetric windows, i.e. windows including more positions upstream than downstream of the cleavage site. This corresponds well with the location of the cleavage site pattern information which is shown as sequence logos (Schneider and Stephens, 1990) in Figure 1. The S-score problem, on the other hand, is best solved by symmetric or approximately symmetric windows.

Although our method is able to locate cleavage sites and discriminate signal peptides from non-secretory proteins with a reasonably high reliability, the accuracy of the cleavage site location is lower than that reported for the original weight matrix method (von Heijne, 1986): 78% for eukaryotes and

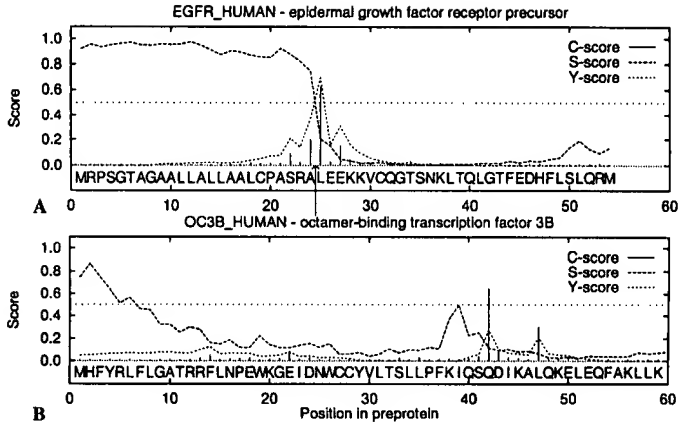

Fig. 2. Examples of network output. The values of the C- (output from cleavage site networks), S- (output from signal peptide networks) and Y-scores (combined cleavage site score, $Y_k = \sqrt{C_i \Delta_d S_i}$) are shown for each position in the sequence. The C- and S-scores are averages over five networks trained on different parts of the data. Note: the C- and Y-scores are high for the position immediately after the cleavage site, i.e. the first position in the mature protein. (A) A successfully predicted signal peptide. The true cleavage site is marked wih an arrow. (B) A non-secretory protein. For many non-secretory proteins, all three scores are very low throughout the sequence. In this example, there are peaks of the C- and S-scores, but the sequence is still easily classified as non-secretory, since the C-score peak occurs far away from the S-score decline and the region of the high S-score is far too short.

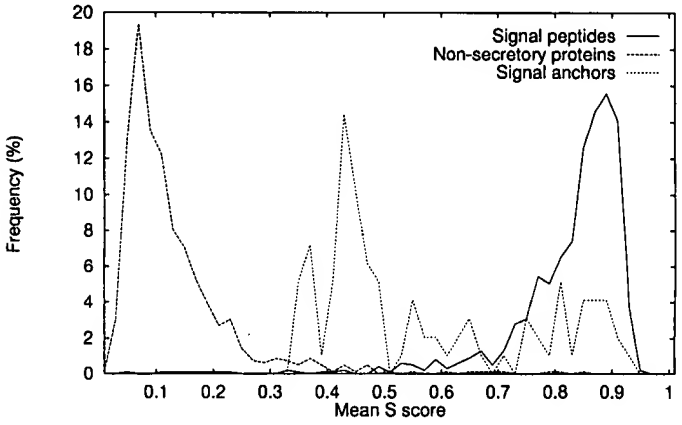

Fig. 3. Distribution of the mean signal peptide score (S-score) for signal peptides and non-signal peptides (eukaryotic data only). 'Non-secretory proteins' refer to the N-terminal parts of cytoplasmic or nuclear proteins, while 'signal anchors' are the N-terminal parts of type II membrane proteins. The mean S-score of a sequence is the average of the S-score over all positions in the predicted signal peptide region (i.e. from the N-terminal to the position immediately before the maximum of the Y-score). The bin size of the distribution is 0.02.

89% for prokaryotes (not divided into Gram-positive and -negative). When the original weight matrix is applied to our recent data set, however, the performance is much lower. This suggests a larger variation in the examples of the signal peptides found since then. It may, of course, also reflect a higher occurrence of errors in our automatically selected data than in the manually selected 1986 set.

In order to compare the strength of the neural network approach to the weight matrix method, we recalculated new weight matrices from our new data and tested the performances of these (results not shown). The weight matrix method was comparable to the neural networks when calculating the C-score, but was practically unable to solve the S-score problem

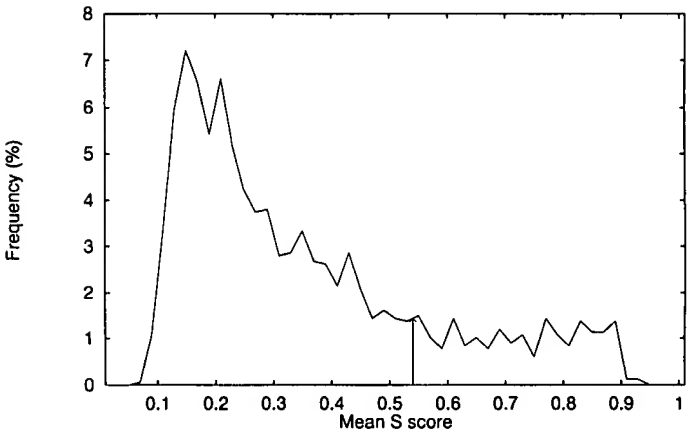

Fig. 4. Distribution of the mean signal peptide score (S-score) for all the predicted *H.influenzae* coding sequences. The mean S-score is calculated using networks trained on the Gram-negative data set. The bin size of the distribution is 0.02. The arrow shows the optimal cut-off for predicting a cleavable signal peptide. The predicted number of secretory proteins in *H.influenzae* (corresponding to the area under the curve to the right of the arrow) is 330 out of 1680 (20%).

and therefore did not provide the possibility of calculating the combined Y-score.

Note that the prediction performances reported here correspond to minimal values. The test sets in the cross-validation have a very low sequence similarity; in fact, the sequence similarity is so low that the correct cleavage sites cannot be found by alignment (Nielsen *et al.*, 1996a). This means that the prediction accuracy on sequences with some similarity to the sequences in the data sets will in general be higher.

The differences between the signal peptides from different organisms are apparent from Figure 1. The signal peptides from Gram-positive bacteria are considerably longer than those of other organisms, with much more extended h-regions, as observed previously (von Heijne and Abrahmsén, 1989). The prokaryotic h-regions are dominated by Leu (L) and Ala (A) in approximately equal proportions and in the eukaryotes they are dominated by Leu with some occurrence of Val (V), Ala, Phe (F) and Ile (I). Close to the cleavage site, the (–3,–1) rule is clearly visible for all three data sets, but while a number of different amino acids are accepted in the eukaryotes, the prokaryotes accept alanine almost exclusively in these two positions. In the first few positions of the mature protein (downstream of the cleavage site) the prokaryotes show certain preferences for Ala, negatively charged (D or E) amino acids, and hydroxy amino acids (S or T), while no pattern can be seen for the eukaryotes. In the leftmost part of the alignment, the positively charged residue Lys (K) [and to a smaller extent Arg (R)] is seen in the prokaryotes, while the eukaryotes show a somewhat weaker occurrence of Arg (barely visible in the figure) and almost no Lys. This corresponds well with the hypothesis that positive residues are required in the n-region where the N-terminal Met is formulated for prokaryotes, but not necessarily for eukaryotes where the N-terminal Met in itself carries a positive charge (von Heijne, 1985).

The difference in structure is reflected in the performances of the trained neural networks (see Table I). Gram-negative cleavage sites have the strongest pattern—i.e. the highest information content—and, consequently, they are the easiest to predict, both at the single-position and at the sequence level. The eukaryotic cleavage sites are significantly more difficult

to predict. Gram-positive cleavage sites are slightly more difficult to predict than the eukaryotic ones, which would not be expected from the sequence logos (Figure 1), since they show nearly as high an information content as the Gram-negative cleavage sites, but the longer Gram-positive signal peptides means that the cleavage sites have to be located against a larger background of non-cleavage site positions. The discrimination of signal peptides versus non-secretory proteins, on the other hand, is better for the eukaryotes than for the prokaryotes. This may be due to the more characteristic leucine-rich h-regions of the eukaryotic signal peptides.

The logos for the human and *E.coli* data sets are not shown, since they show no significant differences from those of the eukaryotes or Gram-negative bacteria respectively. Accordingly, the predictive performance was not improved by training the networks on single-species data sets. On the contrary, the *E.coli* signal peptides are predicted even better by the Gram-negative networks than by the *E.coli* networks (probably due to the relatively small size of the *E.coli* data set). In other words, we have found no evidence for species-specific features of the signal peptides of humans and *E.coli*.

Signal anchors often have sites similar to signal peptide cleavage sites after their hydrophobic (transmembrane) region. Therefore, a prediction method can easily be expected to mistake signal anchors for peptides. In Figure 3, the distribution of the mean S-score for the 97 eukaryotic signal anchors is included. It shows some overlap with the signal peptide distribution. If the standard cut-off of 0.5 is applied to the signal anchor data sets, 50% of the eukaryotic signal anchor sequences are falsely predicted as signal peptides (the corresponding figure for the human signal anchors is 75% when using human networks and 68% when using eukaryotic networks). With a cut-off optimized for signal anchor versus signal peptide discrimination (0.62), we were able to lower this error rate to 45% for the eukaryotic data set. The mean S-score still gives a better separation than the maximal C- or Y-score, which indicates that the pseudo-cleavage sites are in fact rather strong.

However, the pseudo-cleavage sites often occur further from the N-terminal than genuine cleavage sites do. If we do not accept signal peptides longer than 35 residues (this will exclude only 2.2% of the eukaryotic signal peptides in our data set), the percentage of false positives among the signal anchors drops to 28% for the eukaryotic and 32% for the human signal anchors (39% when using eukaryotic networks). When taking this into account, our method does provide a reasonably good discrimination between signal peptides and signal anchors. This has not been reported by any of the earlier published methods for signal peptide recognition.

Scanning the Haemophilus influenzae genome

We have applied the prediction method with networks trained on the Gram-negative data set to all the amino acid sequences of the predicted coding regions in the *Haemophilus influenzae* genome. The distribution of the mean S-score (from position 1 to the position with a maximal Y-score) is shown in Figure 4.

When applying the optimal cut-off value found for the Gram-negative data set, we obtained a crude estimate of the number of sequences with cleavable signal peptides in *H.influenzae*: 330 out of 1680 sequences or approximately 20%. If the maximal S-score is used instead of the mean S-score, the estimate comes out as 28% and with the maximal Y-score it is 14% (distributions not shown). If all three criteria are applied together, leaving only 'typical' signal peptides, we obtain 188 sequences (11%).

Some of the sequences predicted to be signal peptides according to the S-score but not according to the Y-score may be signal anchor-like sequences of type II (single-spanning) or type IV (multispanning) membrane proteins. This hypothesis is strengthened by a hydrophobicity analysis of the ambiguous examples (results not shown). If we apply the slightly higher cut-off optimized for the discrimination of signal anchors versus signal peptides in eukaryotes (0.62) to the mean S-score, the estimate is lowered from 20 to 15%.

On the other hand, some of the sequences predicted to be signal peptides according to the maximal Y-score but not the mean S-score may be the effect of the initiation codon of the predicted coding region having been placed too far upstream. In this case, the apparent signal peptide becomes too long and the region between the false and the true initiation codon will probably not have signal peptide character, thereby bringing the mean S-score of the erroneously extended signal peptide region below the cut-off. This is strengthened by the finding that these ambiguous examples are longer than average and contain more methionines.

In conclusion, we estimate that 15–20% of the *H.influenzae* proteins are secretory. However, a whole-genome analysis like this would be more reliable if combined with other analyses, notably transmembrane segment predictions and initiation site predictions.

Method and data publicly available

The finished prediction method is available both via an e-mail server and a WWW server. Users may submit their own amino acid sequences in order to predict whether the sequence is a signal peptide and, if so, where it will be cleaved. We recommend that only the N-terminal part (say 50–70 amino acids) of the sequences is submitted, so that the interpretation of the output is not obscured by false positives further downstream in the protein.

The user is asked to choose between the network ensembles trained on data from Gram-positive, Gram-negative or eukaryotic organisms. We did not include the networks trained on the single-species data sets in the servers, since these did not improve the performance.

The values of the C-, S- and Y-scores are returned for every position in the submitted sequence. In addition, the maximal Y-score, maximal S-score and mean S-score values are given for the entire sequence and compared with the appropriate cut-offs. If the sequence is predicted to be a signal peptide, the position with the maximal Y-score is mentioned as the most likely cleavage site. A graphical plot in postscript format, similar to those in Figure 2, may be requested from the servers. We strongly recommend that a graphical plot is always used for the interpretation of the output. The plot may give hints about, for example, multiple cleavage sites or erroneously assigned initiation, which would not be found when using only the maximal or mean score values.

The address of the mail server is signalp@cbs.dtu.dk. For detailed instructions, send a mail containing the word 'help' only. The WWW server is accessible via the Center for Biological Sequence Analysis homepage at http://www.cbs.dtu.dk/.

All the data sets mentioned in Table I are available from an FTP server at ftp://virus.cbs.dtu.dk/pub/signalp. Retrieve the file README for detailed descriptions of the data and the format.

The FTP server and the mail server can both be accessed directly from the WWW server.

References

Arrigo,P., Giuliano,F., Scalia,F., Rapallo,A. and Damiani,G. (1991) *CABIOS*, **7**, 353–357.
Bairoch,A. and Boeckmann,B. (1994) *Nucleic Acids Res.*, **22**, 3578–3580.
Brunak,S., Engelbrecht,J. and Knudsen,S. (1991) *J. Mol. Biol.*, **220**, 49–65.
Fleischmann,R. *et al.* (1995) *Science*, **269**, 449–604.
Gierasch,L.M. (1989) *Biochemistry*, **28**, 923–930.
Hirst,J.D. and Sternberg,M.J.E. (1992) *Biochemistry*, **31**, 7211–7218.
Hobohm,U., Scharf,M., Schneider,R. and Sander,C. (1992) *Protein Sci.*, **1**, 409–417.
Ladunga,I., Czakó,F., Csabai,I. and Geszti,T. (1991) *CABIOS*, **7**, 485–487.
Mathews,B. (1975) *Biochim. Biophys. Acta*, **405**, 442–451.
Nielsen,H., Engelbrecht,J., von Heijne,G. and Brunak,S. (1996a) *Proteins*, **24**, 165–177.
Nielsen,H., Engelbrecht,J., von Heijne,G. and Brunak,S. (1997) *Int. J. Neural Sys.*, in press.
Presnell,S.R. and Cohen,F.E. (1993) *Annu. Rev. Biophys. Biomol. Struct.*, **22**, 283–298.
Qian,N. and Sejnowski,T.J. (1988) *J. Mol. Biol.*, **202**, 865–884.
Rapoport,T.A. (1992) *Science*, **258**, 931–936.
Rumelhart,D.E., Hinton,G.E. and Williams,R.J. (1986) In Rumelbart,D., McClelland,J. and the PDP Research Groups (eds), *Parallel Distributed Processing: Explorations in the Microstructure of Cognition. Vol. 1: Foundations*. MIT Press, Cambridge, MA, pp. 318–362.
Schneider,G. and Wrede,P. (1993) *J. Mol. Evol.*, **36**, 586–595.
Schneider,T.D. and Stephens,R.M. (1990) *Nucleic Acids Res.*, **18**, 6097–6100.
Shannon,C.E. (1948) *Bell System Technol. J.*, **27**, 379–423, 623–656.
von Heijne,G. (1983) *Eur. J. Biochem.*, **133**, 17–21.
von Heijne,G. (1985) *J. Mol. Biol.*, **184**, 99–105.
von Heijne,G. (1986) *Nucleic Acids Res.*, **14**, 4683–4690.
von Heijne,G. (1988) *Biochim. Biophys. Acta*, **947**, 307–333.
von Heijne,G. (1990) *J. Membrane Biol.*, **115**, 195–201.
von Heijne,G. and Abrahmsén,L. (1989) *FEBS Lett.*, **244**, 439–446.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: W. James JACKSON
Appl. No.: 09/677,752
Filed: October 02, 2000
For: ***Chlamydia* Protein, Gene Sequence and Uses Thereof**

Confirmation No.: 5261
Art Unit: 1645
Examiner: Vanessa L. FORD
Atty. Docket: 2479.0050000/EJH/C-K

Declaration of W. James Jackson Under 37 C.F.R. § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450
Sir:

I, the undersigned, **W. James Jackson,** residing at 1687 Armistice Way, Marriottsville, Maryland 21104, declare and state as follows:

Credentials.

1. I am the sole inventor of the above-captioned U.S. patent application number 09/677,752, filed October 2, 2000, entitled, "*Chlamydia* Protein, Gene Sequence and Uses Thereof."

2. I am Vice President of Technical Support Department of Emergent BioSolutions Inc. the parent corporation of Emergent Product Development Gaithersburg Inc., ("EMERGENT") the assignee of the present invention. .

3. A current *curriculum vitae* ("C.V.") is appended hereto as **Exhibit A**. As shown in my C.V., I received my Ph.D. degree in Microbiology from the University of Georgia in 1984. From 1984 to 1987, I conducted postdoctoral research at Exxon's Corporate Research and Development Center in Princeton New Jersey and Cold Spring Harbor Laboratories in Long Island New York. Based on my education and experience, I am an expert in infectious diseases and vaccine research and development.

4. I have reviewed the above-identified patent application ("present application"), and the final Office Action dated July 27, 2007 ("the Office Action"). I

have also reviewed Probst *et al.* (U.S. Patent No. 6,432,916, filed April 19, 2000 and issued August 13, 2002) ("Probst"), and Murdin *et al.* (*Infection and Immunity* 61:4406-4414 (1993)) ("Murdin"). I have also reviewed the newly amended claims submitted herewith.

5. It is my understanding that certain claims in the present application have been rejected as being obvious over Probst in view of Murdin. My discussion below of these and other references is offered to assist the Examiner in assessing patentability of the pending claims of the present application.

The Claimed pmpE Vaccine Would Not have been Obvious.

6. Claim 107, as currently amended, is drawn to a vaccine which includes the mature form of the pmpE polypeptide encoded by SEQ ID NO:2 (i.e., the form which is processed and secreted following translation of the naturally-occurring full-length polypeptide). Claim 107 further requires that the claimed vaccine be capable of reducing *Chlamydia trachomatis*-induced infertility in female mice, a very standard and well-accepted model system for testing the efficacy of *Chlamydia* vaccines.

7. The Examiner has concluded that it would have been obvious to one of ordinary skill in the art to combine the Probst and Murdin references to generate the invention of claim 107 as previously claimed. I provide the following discussion to assist the Examiner in evaluating non-obviousness of the invention of claims 107 and 131-146 as presently claimed.

8. As discussed in more detail below, it is my opinion that the invention of claims 107 and 131-146 is non-obvious over the cited references because even if immunogenicity was established, it was extremely unpredictable which *Chlamydia* polypeptides would successfully function as vaccines. Furthermore, it was unexpected that the claimed pmpE vaccine would be cross-protective between serovars. Finally, there has long been an unmet need for an efficacious vaccine against *Chlamydia*.

9. It is my understanding, as explained to me by EMERGENT's patent attorneys, that a claimed invention is non-obvious unless, in view of the literature and general knowledge of scientists in the field, a scientist in the field would have been able to easily predict the claimed invention, and would have had a reasonable expectation that

the invention would work. It is also my understanding that even if these conditions are met, a claimed invention may be determined to be non-obvious upon consideration of factors such as unexpected results, long-felt and unsolved need, commercial success, and/or failed attempts by others.

10. Probst describes cloning of a genomic DNA library of *C. trachomatis* L_2. The reference discloses identified DNA sequences and predicted amino acid sequences of various *Chlamydia* antigens, including the surface proteins pmpA, pmpB, pmpC, pmpD, pmpE, pmpG, pmpH, and pmpI, noting that these, as well as other surface proteins "are believed to be of biological relevance in generating a protective immune response to a Chlamydial infection . . ., and further, that pmpC, pmpD, pmpE, and pmpI contain predictable signal peptides, suggesting they are outer membrane proteins, and therefore, potential immunological targets." *See* Probst, Col. 46, lines 40-51. The pmpE amino acid sequence disclosed in Probst is not the same as SEQ ID NO:2 of the present invention, but does share greater than 98% identity. The only experimental evidence of a protective immune response in Probst is with a DNA vaccine encoding a *C. trachomatis* "SWIB" protein. Aside from noting its likely expression on the cell surface, there is no disclosure singling out pmpE as being more likely of efficacy than any other disclosed antigen. Moreover, Probst fails to provide any experimental data whatsoever regarding the efficacy of any of the disclosed pmp polypeptides as vaccines.

11. Murdin discloses a recombinant poliovirus which expresses a well-characterized neutralization epitope from the major outer membrane protein (MOMP) of *C. trachomatis* serovar A. Murdin showed that the recombinant poliovirus was immunogenic in rabbits, and was capable of neutralizing *C. trachomatis* serovar A *in vitro*. While Murdin demonstrated that the antisera were weakly cross-reactive with serovar C in an ELISA assay, there was no cross-reactivity with serovar B. Finally, Murdin reports that *C. trachomatis* serovar A, pretreated with the same rabbit antisera, had reduced infectivity upon conjuntival inoculation in monkeys. Murdin does not mention any other *Chlamydia* polypeptides, let alone pmpE.

12. *Chlamydia* vaccine research and development is highly complicated and unpredictable as illustrated by numerous publications such as **Exhibits B-H** (described below). For example, a *Chlamydia* protein, while predicted to be encoded by a gene,

may not be expressed in a sufficient level on the outer surface of *Chlamydia*; a *Chlamydia* protein may be antigenic, but may not be immunogenic; a *Chlamydia* protein may be immunogenic, but may not induce an immune response sufficient to provide protection against *Chlamydia* infection; a *Chlamydia* protein may induce a humoral immune response, but may not induce a cell-mediated immune response; and a *Chlamydia* protein may induce a strong immune response (both humoral and cell-mediated), but may not protect an animal against *Chlamydia* infection. In addition, a given *Chlamydia* protein might produce a protective immune response against the serovar from which it was derived, but not against any of the other 18-21 recognized *C. trachomatis* serovars known to cause infection and disease in humans. These factors undermine the necessary objective basis for a reasonable expectation of success that the specific pmpE polypeptide of the present invention could be in fact useful in a vaccine.

13. With respect to the level of pmp polypeptide expression on the surface of *Chlamydia* elementary bodies, the literature demonstrates that among the nine pmp polypeptides known to exist in *C. trachomatis*, only pmpG, pmpH, and (according to Tanzer, a post-filing reference) pmpE are major constituents of the outer membrane of *C. trachomatis*. *See* Mygind *et al. FEMS Microb. Ltts*, 186:163-169 (2000) (**Exhibit B**), and Tanzer and Hatch, *J. Bacteriol.* 183:2686-2690 (2001) (**Exhibit C**). Probst merely discloses various pmp polypeptides predicted to be of interest.

14. Furthermore, prediction, or even demonstration of immunogenicity is no prediction of vaccine efficacy. For example, Su *et al. Vaccine 13*: 1023-1032 (1995) (**Exhibit D**) provides an example of the complex and unpredictable nature of *Chlamydia* vaccine research. In this publication, the investigators hypothesized that a *Chlamydia* polypeptide epitope, which induces both humoral and cell-mediated immune responses, would function to provide a protective vaccine. Indeed, when cynomolgus monkeys were immunized intramuscularly with the polypeptide, the monkeys produced significant levels of neutralizing antibodies. Nonetheless, the monkeys did not acquire protective immunity against cervical challenge with *C. trachomatis*.

15. Likewise, in Batteiger *et al.* (*J. Gen. Microbiol. 139*: 2965-2972 (1993)) (**Exhibit E**), SDS-extracted MOMP of *C. psittaci* induced a strong antibody response in

guinea pigs (as measured by immunoblot analysis), but did not provide protection against *Chlamydia* infection.

16. Similarly, in Pal (*Infect. Immun. 65*: 3361-3369 (1997)) (**Exhibit F**), investigators discovered that a vaccine comprising MOMP (from *C. trachomatis* MoPn) induced a strong humoral immune response and cell-mediated immune response, but failed to confer protection against *Chlamydia* infection, even with the same serovar.

17. Indeed, it is my understanding that aside from our disclosure of cross-protection with pmpG (*see, e.g.*, our co-pending application 08/942,596, submitted in an IDS accompanying this reply as document NPL10), when the present application was first filed most *Chlamydia* vaccine preparations produced only serovar-specific antibodies (*i.e.*, they were directed primarily against the immunodominant and hypervariable serovar-specific MOMP protein and did not cross-react with different *Chlamydia* serovars or species). Additionally, attempts to generate cross-reactive immunogenicity had been only marginally successful as demonstrated by Murdin, described above. *See also* Murdin *et al. Infect. Immun. 63*: 1116-1121 (1995) (**Exhibit G**).

18. In contrast, as shown in the specification, page 66, line 27 to page 67, line 21, the pmpE vaccine claimed in the present application showed a T cell proliferative response, and also cross-protection. The pmpE polypeptide of the present invention induced a strong and uniform antigen-specific T-cell proliferative response. *See* specification at page 66, line 27 to page 67, line 21. In addition, post-filing data surprisingly demonstrated that immunization with a vaccine comprising the serovar L_2 pmpE polypeptide purified as described in Example 6.13 and 6.15 of the present application from the deposited plasmid M15pREP(pQE pmpE Ct)#37 reduced infertility induced by *C. trachomatis* serovar F, in a standard vaginal infectivity and fertility animal models of *C. trachomatis* disease. *See* page 86, line 10 to page 87, line 20 of U.S. Application No. 10/398,248, which claims priority to International Application No. PCT/US01/30345, which is a continuation-in-part application of the present application.

19. Viewed in the context of the unpredictable state of the art relating to *Chlamydia* vaccines, prior to filing of the present application a scientist in the field of *Chlamydia* vaccine research would have had no guidance as to which of the myriad

polypeptides disclosed in Probst might result in a successful, cross-protective vaccine. The scientist would have had to laboriously clone, express, and purify all of the polypeptides, and then blindly test each one in a challenge model, with no assurance that any one of the polypeptides, let alone pmpE, would work. Furthermore, the state of the art was that most existing *Chlamydia* polypeptide vaccines provided only serovar-specific immunogenicity. Thus, the fact that the pmpE L_2 vaccine of the present invention provided protection against a serovar F challenge was surprising. Murdin, showing only serovar-specific passive protection and little or no cross-reactive immunogenicity with a peptide fragment of MOMP, adds little or no reassurance, and no guidance that would direct the scientist to test pmpE.

20. I have further been informed by EMERGENT's patent attorneys that the factors relevant to a non-obviousness analysis include satisfaction of a long-felt need and the failure of prior attempts to address that need. *Chlamydia trachomatis* is a widespread human pathogen. *Chlamydia* genital tract infections are the most frequently reported bacterial sexually transmitted disease in the United States while ocular infections such as trachoma are responsible for causing the most prevalent form of preventable blindness worldwide. *C. trachomatis* has a unique intracellular life cycle, capable of avoiding host immune responses and of establishing chronic infections that often produce serious disease. Statistics show 3-4 million new *Chlamydia* infections occur annually and generate associated health-care costs estimated to exceed $2 billion annually. *See* Peipert, *N Engl J Med. 349*: 2424-30 (2003) (**Exhibit H**). Individuals, particularly, women, with existing urogenitial tract *Chlamydia* infections are at significantly higher risk for contracting other sexually transmitted diseases including HIV. *Chlamydia* infections among pregnant women pose a serious health risk to neonates. Neonatal pneumonia and neonatal conjunctivitis are common complications associated with the delivery of infants through a *Chlamydia* infected birth canal.

21. Although chlamydial infections are susceptible to antibiotic treatment, vaccination is of paramount importance because most infections produce no discernable symptoms thus allowing infections to become established and cause severe disease while escaping diagnosis. Additionally, some groups also experience high rates of re-infection. Yet, in spite of years of efforts by many research groups, as evidenced by the

attached references, a vaccine against human *Chlamydia* infection is still unavailable. The pmpE protein of the present invention has been shown to be effective in protecting against *Chlamydia* infection even across serovars. Thus, the pmpE vaccine of the present invention may address these unmet medical needs by providing an effective *Chlamydia* subunit vaccine.

22. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present application or any patent issued thereon.

Respectfully submitted,

W. James Jackson Ph. D.

Date: October 30th, 2007

W. James Jackson, Ph.D.
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Professional Experience:

Emergent BioSolutions Inc. **June 2007 to Present**
2273 Research Boulevard, Suite 400; Rockville, MD 20850

Vice President, Corporate Technical Support

- **Responsible for new immunobiotic product evaluation, technical / scientific diligence and acquisition**
- **Responsible for M&A technical due diligence efforts**
- **Deputy CSO**

Emergent Product Development Gaithersburg, Inc. **April 2005 to May 2007**
300 Professional Drive; Gaithersburg, MD 20879

Vice President; Product Development, Commercial Products.

- **Responsible for the commercial development of the Antex Biologics vaccine portfolio (Otitis media, Chlamydia, Shigella spp. and Helicobacter pylori).**
- **Responsible for R&D due diligence required for M&A activities.**
- **Responsible for ERDGI Laboratory Operations.**

Antex Biologics Inc. **April 1995 to April 2005**
300 Professional Drive; Gaithersburg, MD 20879

President; Chief Operational Officer (2004 to 2005).

Vice President, Research and Development (2003 to 2004)

Vice President, Research (2000 to 2003).

Director, Research (1997 to 2000).

Director, Molecular Biology (1995 to 1997).

W. R. Grace & Co., Corporate Research Center **August 1987 to February 1995**
7379 Rt. 32; Columbia, MD 21044

Senior Research Molecular Biologist (1990 to 1994).

Research Molecular Biologist (1987 to 1990).

Education:

Postdoctoral Research Fellow
October 1984 to July 1987
Molecular Genetics Group, Exxon Research & Engineering Co., Rt. 22 East, Annandale, NJ 08801.
Barry L. Marrs, Ph.D. & Roger C. Prince, Ph.D. (Sponsoring Scientists)

Philosophy Doctorate (High Honors)
September 1978 to September 1984
Department of Microbiology; University of Georgia, Athens, GA 30602
Anne O. Summers, Ph.D. (Major Professor)

Bachelor of Arts (Summa Cum Laude)
September 1973 to June 1978
College of Arts and Sciences
Microbiology / Biochemistry (Majors)
University of Tennessee, Knoxville, TN 37919.

Vice President, Research

- **Executive Management and Administrative Responsibilities:**

  Reports directly to CEO
  Responsible for all R&D operations
  Executive Committee Member
  Responsible for developing company's IP portfolio
  Functioned as Company's Chief Technical Officer
  Financing / M&A presentations & diligence / Technology acquisitions

- **R&D Responsibilities:**

  Recruited, organized and directed an R&D staff of ~40 scientists into two operating divisions **AntexBiologics** and **AntexPharma.** Responsible for developing and executing company's Biologics and Pharma R&D plans having an annual combined budget of ~$12M.

AntexBiologics:

The mission of AntexBiologics is to prevent or treat select gastrointestinal and sexually transmitted infections and/or their related sequale using vaccine technologies. Program activities range from antigen discovery, biochemical / epidemiological characterization, preclinical immunogenicity and efficacy studies and initial human clinical trials (Phase I/II). Based on clinical outcome, vaccine candidates will be evaluated in later stage clinical studies (Phase III) or out-

licensed. AntexBiologics consists of Microbiology, Molecular Biology, Protein Chemistry, Immunology, and Fermentation groups.

Vaccine Products / Projects Under Development:

TRACVAX - Recombinant *C. trachomatis* subunit vaccine => Status: Phase I

TWARVAX – Recombinant *C. penumoniae* subunit vaccine => Status: Preclinical

GONOVAX - Recombinant *N. gonorrohea* subunit vaccine => Status: Preclinical

HELIVAX – Inactivated *H. pylori* whole cell vaccine => Status: Phase II

HELIVAX II – Augmented *H. pylori* whole cell vaccine => Status: Preclinical

ACTIVAX – Inactivated *S. sonnei* whole cell vaccine => Status: Phase I

Mucosal Adjuvant Development => Discovery / Preclinical

Vice President, Research

AntexPharma:

The mission of AntexPharma is to identify new chemical entities (NCEs) having both broad and narrow spectrum antibacterial activity. Program activities encompass identification/selection of potential antibacterial core structures, SAR-based analogue syntheses, NAACLS-based MIC and MBC analyses, synthetic scale-up, preliminary formulation and in vivo efficacy / bioavailability studies. A lead candidate suitable for topical application will be through initial stage human clinical trials (Phase I/II). Based on clinical outcome, lead candidates will be evaluated in later stage clinical studies (Phase III) or out-licensed. The Pharma unit consists of Antibacterial Research and Organic Synthesis groups.

Anti-infective Products / Projects Under Development:

Heterocyclic alkanes – AP158 => Status: Preclinical / Phase I

Fused lactams => Status: Discovery / Preclinical

Derivativized quinolones => Status: Discovery / Preclinical

Novel Antibacterial Drug Targets => Status: Discovery

- **Development Experience:**

***Helicobacter pylori* – HELIVAX / *Campylobacter jejuni* Vaccines:**

Organized the development of defined, bovine component-free media for use in 300l-scale fermentation of *C. jejuni* and *H. pylori* whole cell vaccines. Directed development of a purification process for the clinical manufacture of a recombinant *H. pylori* subunit vaccine (HP30).

Chlamydia trachomatis – **TRACVAX:**

Directed the development of a purification process for the clinical manufacture of a recombinant *C. trachomatis* subunit vaccine antigen (CT110) and a formulation procedure for mucosal delivery of the CT110 protein.

- **Manufacturing / Clinical Trial Experience:**

Chlamydia trachomatis – **TRACVAX**

Supervised cGMP manufacture (seed banks, fermentation, purification, formulation, fill, release testing) of high value *C. trachomatis* antigen and an ADP-ribosylating mucosal adjuvant for use in Phase I trials.

Designed Phase I safety and immunogenicity trial for the evaluation of the lead antigen candidate. Directed the preparation of TRACVAX Phase I Safety and Immunogenicity IND, Investigator's Brochure and responses to FDA comments. Identified appropriate CROs and clinical trial sites.

Helicobacter pylori – **HELIVAX**

Supervised cGMP manufacture (seed banks, fermentation, inactivation, fill, release testing) of a chemically inactivated *H. pylori* whole cell antigen. Participated in the design of Phase I/II dose-ranging and immunogenicity trials.

Director, Research

- **Management & Administrative Responsibilities:**

Responsible for technical / budgetary management of Smith-Kline Beecham–MicroCarb Human Vaccine R&D discovery and preclinical evaluation programs including:

Project/target identification, project planning (definition of critical path technical milestones/goals); program budget formulation and tracking; project timeline preparation and monitoring; technical resource allocation, IP strategy and staging.

- **R&D Responsibilities:**

***Helicobacter pylori* / *Campylobacter jejuni* / *Shigella spp.* (*S. sonnei, S. flexneri*)**

Development and scale-up of growth conditions to be used to produce virulence-enhanced; whole cell vaccines, preclinical animal model immunogenicity and efficacy evaluation of inactivated whole cell (+/-) mucosal adjuvant vaccines; Phase I & Phase II Clinical Trial design in

collaboration with Naval Medical Research Institute (NMRI, Enteric Disease Program) and SKB.

Vice President, Research

Chlamydia trachomatis / Chlamydia pneumoniae / Neisseria gonorrohea

Identification of subunit vaccine candidate(s) using adhesin-receptor technology (ART); biochemical, epidemiological and immunologic characterization of subunit candidates; production of recombinant proteins in *E. coli* and/or baculovirus; purification / formulation of recombinant antigen for preclinical evaluation; and preclinical animal efficacy evaluation.

Moraxella catarrhalis / Neisseria meningitidis* B / *Haemophilus influenzae

Computational analysis of public/proprietary genomic databases to identify ORFs encoding putative adhesins, hemolysins, type III secretion factors, porins, lipoproteins, pili, redox enzymes (catalase, SOD, PLA), and iron / heme sequestration proteins; production of recombinant proteins in *E. coli* and/or baculovirus; purification / formulation of recombinant antigen for preclinical evaluation; and preclinical animal efficacy evaluation.

Director, Molecular Biology:

- **R&D Responsibilities:**

Responsible for all of company's Molecular Biology activities including:

Genomic database analyses, ORF identification, oligonucleotide design and synthesis, PCR and recombinational cloning, expression (primarily *E.coli*, baculovirus, yeast, CHO), DNA sequencing, genomic library construction, Southern / Northern analyses, knock-out mutant construction, site-directed mutagenesis, subtractive hybridizations, expression vector construction, solubility / activity analysis of expressed protein(s) and recombinant product verification.

In Silico Genomics – SmithKline Beecham Biologics / MCHV

Responsible for cloning/expression and purification of >40 ORFs from select respiratory pathogen genomic databases for in vitro / in vivo evaluation as vaccine candidates:

Director, Molecular Biology:

PCR cloning and expression of membrane-associated ORFs in *E.coli* / Pichia pastoris, RFLP variability analysis of homologue / orthologue ORFs from geographically distinct clinical isolates, DNA sequence analysis of RFLP-variable ORFs, PCR-based genomic walking and sequencing to complete partial ORFs

originating from "high linkage" number databases, ^{3}H-glycerol / ^{3}H-palmitic acid labelling (+/-) globomycin to confirm lipidation of recombinant proteins, construction of novel expression vectors to achieve N-terminal lipidation of recombinant proteins.

VeroTest™

Directed final development of RELISA kit for detecting verotoxin (Shiga-like toxins) producing strains of *E. coli* (e.g.O157:H7), performance / stability testing, GMP manufacture and field evaluations of Research Use Only test.

- **Administrative Responsibilities:**

Patent Liaison:

Assist patent counsel (Pennie & Edmonds) in prosecuting company's U.S. and foreign patent portfolio. Prepared technical responses to Office Actions; represented company at PTO examiner interviews; oversaw/coordinated preparation of patent applications and invention disclosures. Results: 3 issued U.S. patents, 2 Notice of Allowance, 5 new applications filed, and 2 pending invention disclosures.

Radiation Safety Officer:

State RAM license preparation, renewal, and amendments; reviewed and approved protocols involving use of radioactive materials; authorize procurement, receipt, inventory, use and disposal of all licensed materials; coordinate timely health physics monitoring activities (personal and environmental0; decommissioning of RAM use areas.

Chairman, Safety Committee:

Organized company's biological, chemical, and radiological safety program; reviewed safety policies and defined mission, oversee safety training requirements

Senior Research Molecular Biologist:

- **R&D Responsibilities:**

Animal Therapeutics – Bovine Embryo Cloning:

Cloned bovine parathyroid hormone-like protein (bPTHlp) from both cDNA and genomic libraries. Expressed and purified recombinant bPTHlp from *E. coli* and stably transfected methotrexate-amplified CHO-K1 cells. Evaluated bPTHlp for use as a therapeutic treatment for parturient hypocalcemia in cattle via a UMR108 osteosarcoma cAMP-based bioassay.

Identified and employed PCR-based bovine genomic VNTR (dinucleotide) markers for defining origin and genetic parentage of chimeric embryos and fetal tissues during *in utero* development as part of ABS embryo cloning program.

Designed, directed synthesis, purification and evaluated amphipathic synthetic peptides in a fluorescent liposome assay for in vitro cell-cell fusigenic activities. Designed amphipathic synthetic peptides and evaluated via a fluorescent liposome assay for use as an in vitro oocyte-blastomere cell-cell fusigenic agent.

- **Administrative Responsibilities:**

Evaluate External Technologies:

Appraise external intellectual property (technical presentations, scientific notebooks, research reports, patents and patent applications) for "in-house" value and communicate findings and recommendations to senior management, business developers, and patent attorneys.

Research Molecular Biologist:

- **R&D Responsibilities:**

Specialty Chemicals – Artificial Organ Development:

Cloned and expressed a *Vibrio proteolyticus* metalloprotease in *E. coli* and *Bacillus subtilis* as a readily available supply of enzyme for use in bioreactor synthesis of artificial sweeteners. Performed site-directed mutagenesis to increasing thermal stability of a novel *Vibrio* protease to generate enzyme variants having elevated temperature stability profiles.

Research Molecular Biologist:

Initiated development of PCR-based diagnostic assays for detecting and identifying the primary fungal and bacterial agents which cause peritonitis in CAPD patients

Implemented a Western blot system to identify and quantify major serum proteins (albumin, transferrin, immunoglobins, etc.) to define the in vivo adherence and exclusion properties of polysulfone hollow fibers destined for use in an artificial hybrid pancreas.

In collaboration with ANU researchers, developed a PCR-based embryo sexing assay for use in bovine cloning program.

Grantsmanship:

NIH / NIAID – R43 AI3661-01.
Small Business Innovative Research (SBIR) Grant, Phase I.
"Adhesin Vaccine for *Chlamydia trachomatis*"
Funded September 1994 – February 1995 **$74,565**
Principal Investigator

DoD / Office of Naval Research – N00014-96-C-0362.
Small Business Innovative Research (SBIR) Grant, Phase I.
"Rapid Test for Diagnostics of Campylobacter Enteritis and Shigella Dysentery in Operational Ship and Field Environments"
Funded July 1996 – January 1997 **$76,820**
Principal Investigator

NIH / NIAID – R44 AI36617-02.
Small Business Innovative Research (SBIR) Grant, Phase II.
"Development of an Adhesin Vaccine for *Chlamydia trachomatis*"
Funded September 1996 – September 1998 **$838,992**
Principal Investigator

DoD / US Army Medical Research Acquisition Activity – DAMD17-01-C-0026.
Small Business Innovative Research (SBIR) Grant, Phase I.
"Development of a Microbead Whole Cell Delivery System for Oral Immunization Against traveler's Diarrhea."
Funded January 2001 – June 2001 **$68,314**
Principal Investigator

MIPS (Maryland Industrial Partnership Program) – Contract Agreement #2609
University of Maryland, School of Pharmacy
"Airway Mucous Secretion Inhibitors"
Funded August 2000 – July 2001 **$101,685**
Contract Manager

MIPS (Maryland Industrial Partnership Program) – Contract Agreement #2609.28
University of Maryland, School of Pharmacy
"Airway Mucous Secretion Inhibitors / Phase II"
Funded August 2001 – July 2002 **$184,603**
Contract Manager

NIH / NIAID – AI56452-02.
"Novel Adjuvants for Biodefense Vaccines"
Funded September 2003 – August 2005 **$523,116**
Co-Principal Investigator

Issued Patents:

USPTO 6,642,023. Chlamydia protein, gene sequence and uses thereof.
Inventors: Jackson; W. James and Pace; John L.; Assignee: Antex Biologics, Inc.

USPTO 6,693186. Neisseria meningitidis protein, nucleic acid sequence and uses thereof.
Inventors: Jackson; W. James and Harris; Andrea M.; Assignee: Antex Biologics, Inc.

USPTO 6,756,493. Neisseria species protein, nucleic acid sequence and uses thereof.
Inventors: Jackson; W. James and Harris; Andrea M.; Assignee: Antex Biologics, Inc.

USPTO 6,887,843. Chlamydia trachomatis proteins and uses thereof.
Inventors: Jackson; W. James and Pace; John L.; Assignee: Antex Biologics, Inc.

Published Patent Applications:

"Cloning and Expression of the Bovine Parathyroid Hormone Related Protein and Use as a Prophylactic Treatment for Parturient Paresis in Dairy Cattle." Applicant: W.R. Grace & Co.-Conn.; Inventor: Washington James Jackson: PTO Application Number: 860,708.

"Neisseria meningitidis Polypeptide Gene Sequences and Uses Thereof." Applicant: Antex Biologics Inc.; Inventors: W. James Jackson and Andrea M. Harris; US 09/388,089.

"Neisseria spp. Polypeptide Gene Sequences and Uses Thereof." Applicant: Antex Biologics Inc.; Inventors: W. James Jackson and Andrea M. Harris; US 09/388,090.

"Chlamydia spp. Polypeptide, Gene Sequences and Uses Thereof": Applicant: Antex Biologics Inc.; Inventors: W. James Jackson and John L. Pace; US 08/866,592.

"Chlamydia spp. Polypeptides, Gene Sequences and Uses Thereof": Applicant: Antex Biologics Inc.; Inventors: W. James Jackson; US 09/459,032.

"Helicobacter pylori Polypeptide, Gene Sequences and Uses Thereof": Applicant: Antex Biologics Inc.; Inventors: Jing-Hui Tian, W. James Jackson and Richard I. Walker; US 08/639,936.

"Novel 2-Aryloxyphenol Derivatives as Antibacterial Agents"; Applicant: Antex Biologics Inc.; Inventors: Liren Huang, Wei-Tong Wang, Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 09/388,090.

"4-Subsituted 2-Aryloxyphenol Derivatives as Antibacterial Agents" Applicant: Antex Pharma Inc.; Inventors: Liren Huang Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 11/214,772.

2-(2 Or 4-Subsituted Aryloxy)-Phenol Derivatives as Antibacterial Agents": Applicant: Antex Pharma Inc.; Inventors: Liren Huang Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 11/289,362.

Novel 2-Heterooryloxy-Phenol Derivatives as Antibacterial Agents": Applicant: Antex Pharma Inc.; Inventors: Liren Huang Alenka G. Tomazic, Joanna M. Clancy and W. James Jackson; US 11/320,755.

Publications:

1. Jackson, W.J. and A. O. Summers. 1982. Polypeptides Encoded by the Mercury-Resistance (mer) Operon. J.Bacteriol. 149:479-487.

2. Jackson, W. J. and A. O. Summers. 1982. Biochemical Characterization of the HgCl2-Inducible Polypeptides Encoded by the mer Operon of Plasmid R100. J.Bacteriol. 151:962-970.

3. Barrineau, P., P. Gilbert, W. J. Jackson, C. Slater-Jones, A. O. Summers, and S. Wisdom. 1985. The DNA Sequence of the Mercury-Resistance Operon of the IncFII Plasmid NR1. J.Mol.Appl.Gen. 2:601-619.

4. Barrineau, P., P. Gilbert, W. J. Jackson, C. Slater-Jones, A. O. Summers, and S. Wisdom. 1985. The Structure of the mer Operon, p. 701-718. In D. R. Helinski and S. N. Cohen (eds.), Plasmids in: Bacteria. Plenum Press, New York.

5. Jackson, W. J. Jackson, R. C. Prince, and B. L. Marrs. 1986 Energetic and Topographic Properties of a B875 Light-Harvesting Mutant of Rhodopseudomonas capsulata.
Biochem. 25:8440-8446.

6. Prince, R. C. and W. J. Jackson. 1986. The Role of the Light-Harvesting I Antenna Proteins in the Correct Insertion of the Photochemical Reaction Center of Rhodobacter capsulatus and Rhodobacter sphaeroides. Progress in Photosynthesis Research (Biggins, J., ed.)
Martinus Ni jhoff, Dordrecht 4:721-724.

7. Jackson, W. J. and R. C. Prince. 1986. Genetic and Sequence Analysis of a Rhodobacter capsulatus Mutant Unable to Properly Insert the Photochemical Reaction center into the Photosynthetic membrane. Progress in Photosynthesis Research (Biggins, J., ed.) Martinus Nijhoff, Dordrecht 4:725-728.

8. Heltzel, A. G., W. J. Jackson, D. A. Gambill, and A. O. Summers. 1987. Overexpression and DNA-Binding Properties of the mer-Encoded Regulatory Protein from Plasmid NR1. (Tn21). J.Bacteriol. 169:3379-3384.

9. Jackson, W. J. , P. T. Kiley, S. Kaplan, and R. C. Prince. 1897. On the Role of the Light-Harvesting B880 Apparatus in the Correct Insertion of the Reaction Center of Rhodobacter capsulatus and Rhodobacter sphaeroides. FEBS.Lett. 215:171-174.

10. Sesno, J.A., R. M. Overton, and W. J. Jackson. 1994. Genomic and cDNA Cloning of the Bovine Parathyroid Hormone-Related Protein (bPLP). Manuscript submitted Gene.

11. Stice, S. L., N. S. Strelchenko, W. J. Jackson, C. A. Keefer, and L. W. Matthews. 1994. Bovine Embryonic Stem Cell Lines: Demonstration of Pluripotency Following Nuclear Transfer. Manuscript submitted, Theriogenology.

12. McKenzie, R. L., R. I. Walker, G. S. Nabors, C. Carpenter, G. Gomes, E. Forbes, J. H. Tian,, H. H. Yang, J. L. Pace, W. J. Jackson, and A. L. Bourgeois. 2004. Safety and Immunogenicity of an Oral, Inactivated, Whole-Cell Vaccine for Shigella sonnei: Preclinical Studies and a Phase I Trial.

Abstracts:

1. Jackson, W. J. and A. O. Summers. 1979. Proteins Synthesized in minicells harboring cloned mer DNA. Abstracts of the Third Annual Mid-Atlantic Regional Extrachromosomal Elements Meeting. Plasmid. 3:235-242.

2. Jackson, W. J. and A. O. Summers. 1980. Genetic and polypeptide analysis of the mer operon. Abstracts of the Annual Meeting of the American Society for Microbiology, H-35, p.341.

3. Jackson, W. J., F. A. Bohlander, and A. O. Summers. 1981. The mer operon: Polypeptides and a promoter. Annual Meeting of the Genetics Society of America, June 1981.

4. Jackson, W. J. and A. O. Summers. 1982. The relation of the HgCl2-inducible R-factor membrane proteins to HgCl1-resistance in Escherichia coli. Abstracts of the Annual Meeting of the Society for Microbiology, CCI-113, p.526.

5. Jackson, W. J. and R. C. Prince. 1986. Genetic and sequence analysis of a Rhodopseudomonas capsulata mutant deficient in the LHI complex. Abstracts of the Eastern Regional Photosynthesis Conference, March 1986.

6. Jackson, W. J., R. C. Prince, and B. L. Marrs. 1986. Characterization of a light-harvesting I (LHI) mutant of Rhodopseudomonas capsulata. Abstracts of the Annual Meeting of the American Society for Microbiology, K-101, p.210.

7. Jackson, W. J. and R. C. Prince. 1987. The role of the light-harvesting I antenna proteins in the correct insertion of the photochemical reaction center. Abstracts of the VII International Congress on Photosynthesis, 307, p. 197.

8. Jackson, W. J. and R. C. Prince. 1988. Spectroscopic analysis of two Rhodobacter capsulatus B875 site-directed mutants. Abstracts of the Annual Meeting of the American Society for Microbiology, K-150, p.231.

9. Jackson, W. J. and J. A. Sesno. 1992. Cloning and expression of a bovine parathyroid hormone-like protein (bPLP) cDNA from lactating mammary tissue. Abstracts of the Annual FASEB Meeting, FASEB Journal 6 (4), p. 1438.

10. Welch, G. R., W. J. Jackson and L. A. Johnson 1993. Single cell sorting and PCR sexing analysis to confirm separation of X- and Y-chromosome bearing bovine sperm. Society of Analytical Cytology, Annual Meeting. Cytometry, Supplement 6, p. 26.

11. Lanza, R.P., W. J. Jackson, J. M. Wolfrum, B. A. Solomon, and W. I. Chick. 1993. Immunoperfusion properties of the hybrid artificial pancreas. Artificial Organs, 17 (6), p. 429.

12. Stice, S. L., N. Strelchenko, J. Betthauser, B. Scott, W. J. Jackson, V. A. David, C. Keefer, and L. A. Matthews. !994. Bovine pluripotent embryonic cells contribute to nuclear transfer and chimeric fetuses. Theriogenology 41, p. 301.

13. Tucker, K. D., L. Plosila, W. J. Jackson, L. MacDonald, and R. P. Johnson. 1995. VeroTest, a receptor-based ELISA for detecting Shiga-like toxins. Abstracts of the Annual Meeting of the American Society for Microbiology, P-76, p. 395.

14. Jackson, W. J., J-F., Maisonneuve, R. Taylor, J-H. Tian, A. Harris, and H-H. Yang. 2001. Immunization with a High Molecular weight Protein (pmpG) from Chlamydia trachomatis Confers

Heterotypic Protection Against Infertility. Abstracts of the Annual Meeting of the American Society for Microbiology, E-23, p. 197.

15. Maisonneuve, J-F., R. Taylor, J-H. Tian, A. Harris, H-H. Yang, and W. J. Jackson. 2001. A Vaccine Comprising a High Molecular Weight Protein (pmpG) Elicits a Strong T-Cell Response and Confers Protection Against Infertility Resulting from a Chlamydia trachomatis Genital Challenge. Abstracts of the International Society on Sexually Transmitted Diseases, P-113, p233.

16. Taylor, R. E., J-H Tian, K. Johnson, X. Ding, N. Chang, M. A. Rhodes, R. Harris and W. J. Jackson. 2002. Mucosal Immunization with Recombinant pmpE from Chlamydia trachomatis Serovar L2 Confers Protection Against Serovar F-Induced Infertility. Abstracts of the Annual Meeting of the American Society for Microbiology, E-53, p. 182.

17. Robert Taylor, Joanna Clancy, Liren Huang, Alenka Tomazic, Lisa Koterwas, and W. James Jackson. Efficacy of Topical AP-158 in a *S. aureus* Murine Wound Infection Model. 8th International Antibacterial Drug Discovery and Development Summit. Proceedings. March 24th-25th Hyatt Regency Princeton, NJ.

18. Identification of a protective 30kDa antigen of *Helicobacter pylori*
R. Keefe, G. Nabors, J. Tian, R. Walker, Y. Feng, R. Harris, J. Jackson; Antex Biologics, Gaithersburg, MD.

19. Mucosal immunization with a recombinant *Chlamydia trachomatis* high molecular weight protein protects mice against heterotypic genital infection
H. Lu, A. M. Harris, G. S. Nabors, W. J. Jackson;
Antex Biologics; Gaithersburg, MD 20879

20. Towards the Development of a *Chlamydia trachomatis* Subunit Vaccine.
Hang Lu, Gary Nabors, Steve Roberts, Huei-Hsiung Yang, Yang Feng
and W. James Jackson*
Antex Biologics Inc., Gaithersburg, MD 20879.

21. Safety and Immunogenicity of an Oral, Inactivated, Whole-Cell Vaccine for Shigella sonnei. McKenzie, R. L., G. S. Nabors, C. Carpenter, G. Gomes, E. Forbes, A.L. Liss, W. J. Jackson, J. H. Tian,, H. H. Yang, R. I. Walker and A. L. Bourgeois. 2004. VED, May 2004.

22. The Immunogenicity and Protective Capacity of BioThrax® Are Significantly Enhanced by CPG 7909 M. GU[1], J. CLANCY[1], P. HINE[1], C. BOTEZAN[1], L. SIMON[1], B. KINTNER[1], L. BONDOC[1], I. SIM[2], C. NIELSEN[3], G. NABORS[1], W.J. JACKSON[1], L. GIRI[1]
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Membrane proteins PmpG and PmpH are major constituents of *Chlamydia trachomatis* L2 outer membrane complex

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Abstract

The outer membrane complex of *Chlamydia* is involved in the initial adherence and ingestion of *Chlamydia* by the host cell. In order to identify novel proteins in the outer membrane of *Chlamydia trachomatis* L2, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. By silver staining of the protein profile, a major protein doublet of 100–110 kDa was detected. In-gel tryptic digestion and matrix-assisted laser desorption/ionization mass spectrometry identified these proteins as the putative outer membrane proteins PmpG and PmpH. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Chlamydia*; Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Mass spectrometry; Membrane protein

1. Introduction

The different species of the genus *Chlamydia* are obligately intracellular bacteria, causing a wide range of diseases in both humans and animals [1]. The recent genome sequencing of *Chlamydia trachomatis* serovar D and *C. pneumoniae* has provided a blueprint of potential genes involved in the pathobiology of *Chlamydia* [2,3]. As many as 225 of the 894 (28%) likely protein encoding regions in *C. trachomatis* did not show any significant homology to other sequences deposited in GenBank. In *C. pneumoniae* another 186 (of 1073) likely protein encoding regions were identified with no homology to known sequences in other organisms, including *C. trachomatis* serovar D. Among the most interesting discoveries was the existence of a larger group of potential outer membrane proteins (Pmp). A total of nine genes in *C. trachomatis* and 21 genes in *C. pneumoniae* were identified. The Pmp proteins are heterologous in sequence (9–42% amino acid identity between Pmp proteins of *C. trachomatis* and predicted molecular mass from 96000 to 187000 kDa). They all encode a carboxy-terminal phenylalanine, a repeated GGAI motif and all protein encoded sequences contain predicted cleavable signal peptides, suggesting a localization of the expressed proteins in the outer membrane of *Chlamydia* [4]. It is likely that they play an important role in *Chlamydia* structural, functional or antigenic polymorphism.

The *Chlamydia* outer membrane complex (COMC) is prepared by sarcosyl extraction of infectious *Chlamydia* elementary bodies (EB) [5]. It contains a surface-exposed genus-specific lipopolysaccharide, but the structural stability of the complex is attributed to extensive intermolecular disulfide bond cross-linking of the major outer membrane protein (MOMP, 37–40 kDa), outer membrane protein 2 (Omp2, 57–62 kDa) and outer membrane protein 3 (Omp3, 12–15 kDa). All three proteins are generally well conserved among *Chlamydia* species, but MOMP contains variable regions detected on the surface of *C. psittaci* and *C. trachomatis* [6]. However, data suggest that there is no association between *Chlamydia* pathogenicity/host cell preference and the MOMP amino acid sequence of various *Chlamydia* species and serovars [7].

In 1996, three Pmp proteins were identified in outer membrane preparations of an ovine abortion strain of *C. psittaci* [8]. They were shown to be highly immunogenic in post-abortion sheep sera and one of these proteins was

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immuno-accessible on the surface of *C. psittaci* [9]. In analogy to these studies, two Pmp proteins have been identified as components of the outer membrane complex in *C. pneumoniae* [10]. The presence of highly variable Pmp proteins in the outer membrane of different *Chlamydia* species indicates a putative role of this protein family in antigenic diversity and/or pathogenicity of each *Chlamydia* species.

The question whether Pmp proteins are expressed in the *C. trachomatis* outer membrane complex has not been answered. We here report the identification of Pmp proteins in *C. trachomatis* L2, the agent of severe systemic human infections (lymphogranuloma venereum, LGV) [1]. The proteins were isolated from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) separation of outer membrane proteins and identifications were performed by peptide mass mapping. This was done by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), followed by matching the data with those available from the genome sequence database [2,12].

2. Materials and methods

2.1. Cultivation and purification of C. trachomatis

C. trachomatis L2 (LGV-II/434/Bu) was cultivated in confluent monolayers of HeLa 229 epithelial cells as previously described [13]. After 48 h of infection, cells were harvested, disrupted and *Chlamydia* EB were purified on an Isopaque gradient (Nyegaard Diagnostica, Norway).

2.2. Purification of C. trachomatis outer membrane complex

Outer membrane complexes were prepared essentially as originally described [5]. Purified EB were resuspended in 2% (w/v) sarcosyl (in phosphate buffered saline (PBS, pH 7.4), 5 mM $MgCl_2$, 60.2 U ml^{-1} benzoase, and 0.14 U ml^{-1} RNase). The suspension was briefly sonicated and incubated for 30 min at 37°C. Sample was centrifuged for 30 min at 20 000 × *g*. Pellet was resuspended twice in 2% (w/v) sarcosyl (in PBS, 1.5 mM EDTA (ethylenediaminetetraacetic acid)), sonicated and pelleted by centrifugation for 30 min at 20 000 × *g*. The pellet was finally resuspended in PBS buffer and stored at −70°C.

2.3. SDS–PAGE

Protein content of COMC was estimated using a bicinchoninic acid assay system (BCA™, Pierce, Rockford, IL, USA). Samples were dissolved in SDS sample buffer (50 mM Tris–HCl [pH 8.8], 10% (w/v) glycerol, 2.3% (w/v) SDS, 5% (w/v) β-mercaptoethanol) and boiled for 5 min. Proteins were then separated by SDS–PAGE using a 5–25% continuous polyacrylamide gradient gel.

2.4. Silver and Coomassie brilliant blue staining of SDS–PAGE-separated proteins

Gel slabs were silver stained as previously described [14]. Coomassie brilliant blue staining of proteins was performed by soaking the gel in 1% (w/v) Coomassie brilliant blue R-250 (dissolved in 10% (v/v) acetic acid, 40% (v/v) methanol and 50% (v/v) distilled water) for 45 min followed by destaining in 5% (v/v) methanol and 7.5% (v/v) acetic acid (3 h).

2.5. In-gel tryptic digestion of proteins and peptide concentration

Sample preparation for protein identification by MS-MALDI-time-of-flight (TOF) was carried out essentially as described [12,15]. The flow diagram in Fig. 2 depicts the strategy applied. Proteins in gel plugs were dehydrated, reduced and alkylated prior to trypsin digestion. Custom chromatographic columns were used for desalting and concentration of the resulting peptide mixture. Peptides were eluted with matrix solution and deposited directly onto the mass spectrometry probe [12].

2.6. MALDI-MS and protein identification

A Bruker REFLEX MALDI-TOF mass spectrometer (Bruker-Franzen Analytik GmbH, Germany) equipped with the SCOUT source and variable detector bias gating was employed for mass analysis of peptide mixtures in positive ion reflector mode [12]. Tryptic peptide derivatives from tryptic autodigestion were used to calibrate each spectrum to obtain a high mass accuracy. Protein identification was accomplished by submitting the measured monoisotopic tryptic peptide masses to a comprehensive sequence database (Protein Prospector, http://www.prospector.ucsf.edu).

2.7. Protein accession numbers

AAC15922 (POMP90A), AAC15921 (POMP91A), AAC15923 (POMP91B), AAB18188 (POMP98), CAA04672 (Omp4), CAB37071 (Omp5), AAC68469 (PmpG), AAC68470 (PmpH).

3. Results

3.1. Staining of COMC proteins upon SDS–PAGE separation

A different staining profile of *C. trachomatis* L2 COMC proteins was seen using traditional Coomassie brilliant blue and silver staining (Fig. 1, lanes 2 and 3). Most striking was the divergent staining of high molecular mass proteins (approximately 105 kDa and 150 kDa). These

Fig. 1. SDS–P. *chomati* COMC Omp3). lecular

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3.2. Ia

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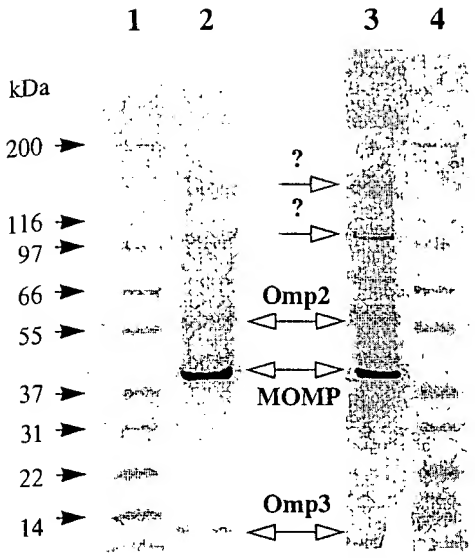


Fig. 1. Staining of *C. trachomatis* L2 COMC proteins upon 5–25% SDS-PAGE separation. Lane 2: Coomassie brilliant blue-stained *C. trachomatis* L2 COMC (2.5 μg). Lane 3: silver-stained *C. trachomatis* L2 COMC (0.5 μg). Known proteins are marked (MOMP, Omp2 and Omp3). Lanes 1 and 4 are identical molecular mass standards, with molecular mass sizes indicated to the left of the figure.

protein bands, easily detectable by silver staining (Fig. 1, lane 3), were only weakly stained by Coomassie brilliant blue (Fig. 1, lane 2).

As illustrated in Fig. 2A, all bands of potential Pmp proteins (above 90 kDa) were excised from a silver-stained gel. Samples were prepared, digested by trypsin, analyzed by MALDI-MS, and identified according to the procedure shown in Fig. 2B.

3.2. Identification of PmpH

The tryptic mass fingerprint of the excised 103-kDa protein is shown in Fig. 3. By a stringent peptide mass search (50 ppm) of the measured tryptic fragments against the database of theoretically trypsin-digested proteins, *C. trachomatis* PmpH was unambiguously identified. Twenty-four tryptic peptides were assigned to this protein. The secondary search result assigned 13 peptides to an unrelated protein of *Serratia marcescens*. By lowering the stringency to 100 ppm, another 10 tryptic peptides could be assigned to *C. trachomatis* PmpH covering a total of 31% of the protein sequence (Fig. 3). Four of the identified peptides (13′, 15′, 18′, 28′) were modified (Met-Ox, Cys-Am or phosphorylated) derivatives of already identified tryptic fragments (13, 15, 18, 28). The serovar L2 ortholog of PmpH is not included in the database and was retrieved from the *Chlamydia* genome project (http://chlamydia-www.berkeley.edu:4231/). As expected, all but three identified tryptic fragments were located in regions with 100% identity between serovars D and L2. In one tryptic fragment (peptide 22, 2150.0677 Da, Fig. 3) the methionine residue (131.0405 Da) is substituted by valine (99.0684 Da) in *C. trachomatis* serovar L2 PmpH. The adjusted theoretical tryptic peptide mass (2117.9932 Da) was well within the search criteria of another experimentally detected tryptic peptide mass (2118.0093 Da, peptide I, Fig. 3). Regarding tryptic peptide mass 28 (2807.4154 Da) and 29 (2832.5062 Da) the substitution (proline, 97.0528 Da to leucine, 113.0840 Da) still allowed a positive identification of the peptide. When a theoretical digestion of L2 PmpH was performed, another three *C. trachomatis* L2-specific tryptic peptides could be assigned in the peptide mass spectrum (Fig. 3, peptides marked II–IV). The theoretical tryptic peptide (GIVLFKDNEGGIFFR, 1711.9172 Da) could easily be assigned to a tryptic peptide in the mass spectrum (peptide II, 1711.8960 Da). Another tryptic peptide (SYVDIKGTETVVYWETAYGYSVHR, 2823.3630 Da) was assigned to the experimentally detected tryptic peptide III (2823.4165 Da, Fig. 3). The last *C. trachomatis* L2-specific tryptic fragment that could be assigned in the spectrum is a methoxylated peptide (GmVPPNTSNTLYLTWRPASNYGEYR) with a theoretical tryptic peptide mass of 2903.3787 Da (peptide IV in Fig. 3, 2903.5310 Da).

3.3. Identification of PmpG

The 107-kDa band in the *C. trachomatis* L2 COMC was excised from the gel slab and subjected to MS-MALDI for identification of peptide masses upon tryptic digestion. The peptide mass fingerprint is depicted in Fig. 4. Using

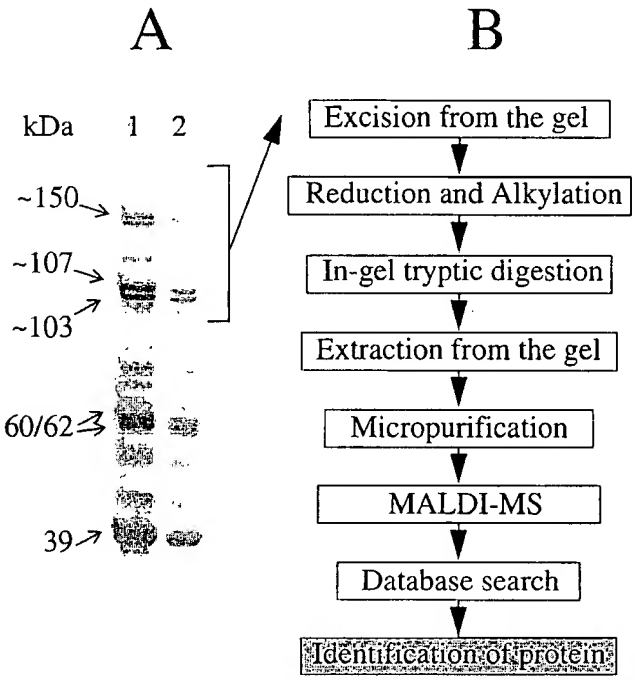


Fig. 2. A: Lanes 1 and 2 are both silver-stained SDS–PAGE-separated *C. trachomatis* L2 COMC (2.5 and 0.25 μg, respectively). The approximate molecular mass of MOMP, Omp2 and unknown high molecular mass proteins is indicated to the left. B: Flow diagram depicting the identification of novel proteins in COMC by MALDI-MS.

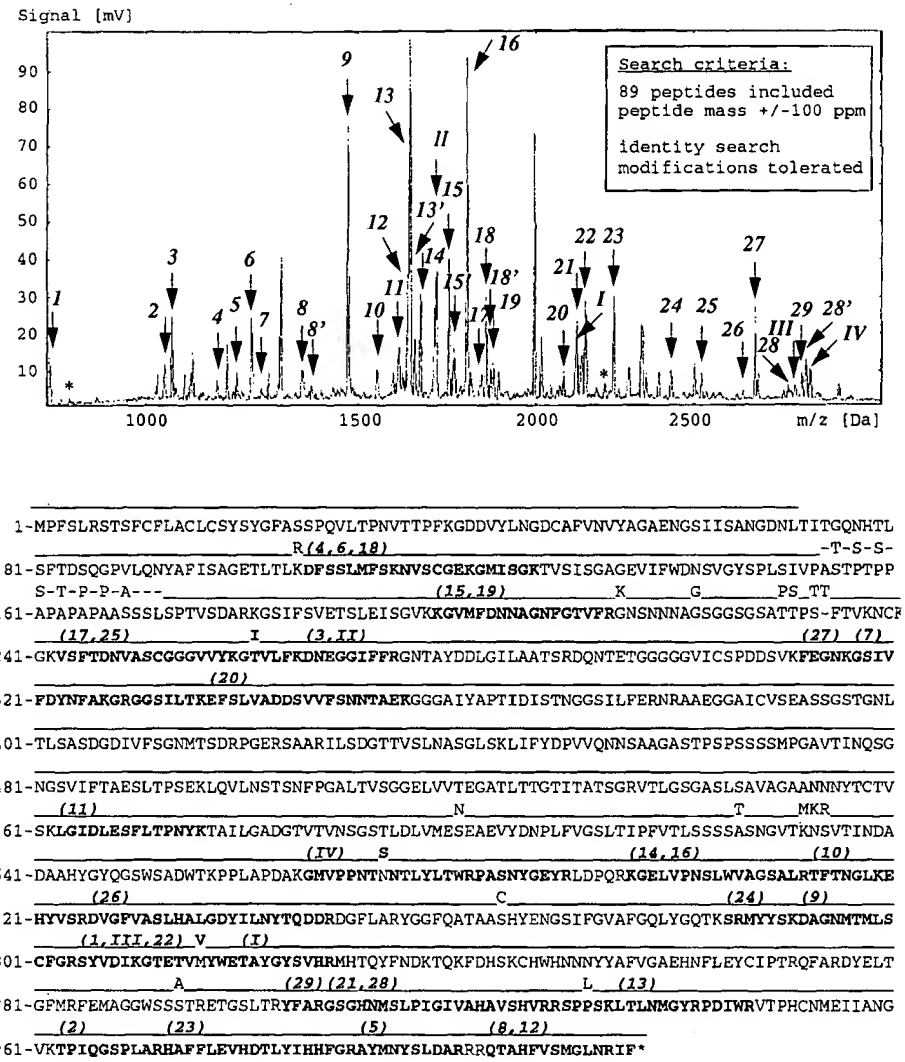


Fig. 3. Peptide mass fingerprint identifying *C. trachomatis* PmpH. Arrows indicate masses assigned to the protein sequence of PmpH. The *C. trachomatis* D PmpH is reproduced below the spectrum with identified peptides outlined in bold. Above the sequence is indicated the homolog *C. trachomatis* L2 PmpH protein (solid line indicates an identical amino acid in *C. trachomatis* L2 PmpH). Identified masses are: (Da) 804.3077 (1), 1039.5316 (2), 1054.4432 (3), 1157.5241 (4), 1203.4912 (5), 1237.5196 (6), 1260.5501 (7), 1360.6334 (8), 1376.6149 (8′), 1475.6997 (9), 1551.7365 (10), 1609.8145 (11), 1619.8764 (12), 1634.8234 (13), 1650.8146 (13′), 1668.8760 (14), 1711.8960 (II), 1745.7872 (15), 1761.7886 (15′), 1796.9868 (16), 1839.9753 (17), 1851.9051 (18), 1865.9405 (18′), 1873.8965 (19), 2071.9842 (20), 2118.0093 (I), 2139.1187 (21), 2150.0677 (22), 2239.1153 (23), 2423.1182 (24), 2565.3799 (25), 2582.2569 (26), 2707.2169 (27), 2807.4154 (28), 2823.4165 (III), 2832.5062 (29), 2887.4927 (28′), 2903.5310 (IV). The asterisks indicate tryptic autolytic fragments (Da 842.5100 and 2211.100).

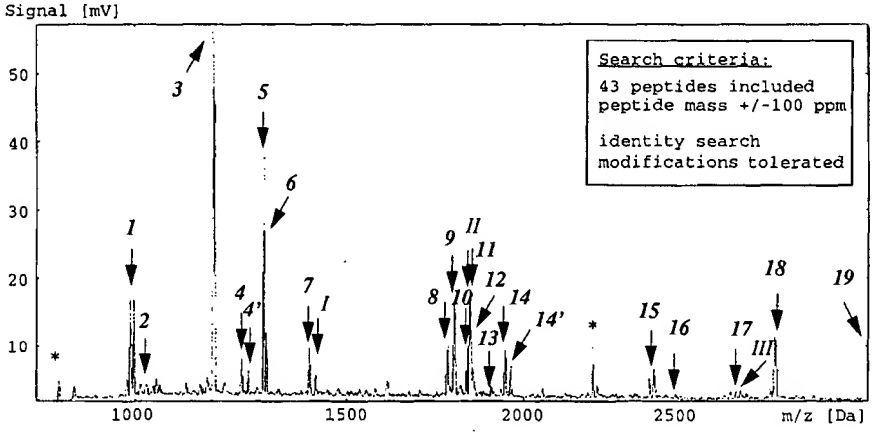
stringent criteria (50 ppm) we identified the protein as PmpG, and 17 peptides were assigned to this protein. The secondary search result assigned 11 peptides to an unrelated protein of *Saccharomyces cerevisiae*. By lowering the stringency to 100 ppm, another four tryptic peptides could be assigned to *C. trachomatis* PmpG covering a total of 27% of the protein sequence (Fig. 4). Methoxylated derivatives of two peptides (4 and 14, Fig. 4) were also detected (4′ and 14′, Fig. 4). The serovar L2 homolog of PmpG is not included in the database used and was retrieved from the *Chlamydia* genome project (http://chlamydia-www.berkeley.edu:4231/). All but three identified tryptic fragments were located in regions with 100% identity between serovars D and L2. In the last three tryptic fragments (12, 16, 17) a lysine residue (128.0950 Da) is replaced by a glutamine residue (128.0586) in *C. trachomatis* L2. This substitution does not affect the identification of the tryptic fragment. Three *C. trachomatis* L2 PmpG-specific peptides could be identified in the peptide mass spectrum (marked I–III in Fig. 4). The experimentally determined tryptic peptide of 1419.7517 Da (marked 'I' in Fig. 4) could be identified as the theoretical peptide SGHLLNLSVPVGVK (1419.8324 Da). A verification of this assignment was seen by the assignment of peptide SGHLLNLSVPVGVKFDR (1838.0288 Da) to an experimentally determined fragment (1838.0008 Da, peptide II in Fig. 4). Lastly, the 2697.4815-Da tryptic peptide could be identified as the *C. trachomatis* L2-specific PmpG peptide III (AGHQILFNDPIEMANGNNQPAQSSK. 2697.2691 Da).

The analysis of other high molecular mass proteins in *C. trachomatis* L2 COMC revealed no other potential membrane-associated proteins. Bands of approximately 150 kDa were found to be contaminants of the outer membrane complex, namely *C. trachomatis* RNA polymerase β and β′ (data not presented).

4. Discussion

The present study is the first to identify Pmp proteins in the outer membrane of *C. trachomatis*. PmpG and PmpH were identified as major constituents of this complex, whereas none of the seven other predicted Pmp proteins could be detected in this complex using the methods applied (SDS–PAGE, silver staining and MS-MALDI-TOF). This was achieved even though amino acid substitutions are present in sequences of the *C. trachomatis* L2 Pmp proteins studied compared to the *C. trachomatis D* genome available in the database. A recent report indicates transcription of mRNA from all nine *pmp* genes in *C. trachomatis* L2 [16]. The level of transcription of individual *pmp* genes was, however, not quantitated in the study [16]. Our results are consistent with studies reported on both *C. pneumoniae* and *C. psittaci*. In both species, a subset of potential Pmp proteins were also readily detected in the *Chlamydia* outer membrane. These homologs were originally named Omp4, Omp5 (*C. pneumoniae*) and POMP90A/B, POMP91A/B (*C. psittaci*) [8,10]. Interestingly, all the identified expressed *pmp* genes are located in clusters [10,11]. This is also the case in the present study, as PmpG and PmpH are situated closely together in the *C. trachomatis* genome [2].

Little is known about the function or topology of the Pmp proteins in *Chlamydia*. The inter- and intra-species variability of these proteins is striking, with respect to



1-MQTSFHKFFLSMILAYSCCSLSGGGYAAEIMIPQGIYDGETLTVSFPYTVIGDPSGTTVPSAGELTLKNLDNSIAALPLS
81-CFGNLLGSFTVLGRGHSLTFENIRTSTNGAALSDSANSGLFTIEGFKELSFSNCNSLLAVLPAATTNNGSQTPTTTSTPS
161-NGTIYSKTDLLLLNNEKFSFYSNLVSGDGGAIDAKSLTVQGISKLCVFQENTAQADGGACQVVTSFSAMANEAPIAFIAN
241-VAGVRGGGIAAVQDGQQGVSSSTSTEDPVVSFSRNTAVEFDGNVARVGGGIYSYGNVAFLNNGKTLFLNNVASPVYIAAE
321-QPTNGQASNTSDNYGDGGAIFCKNGAQAAGSNNSGSVSFDGEGVVFFSSNVAAGKGGAIYAKKLSVANCGPVQFLGNIAN
401-DGGAIYLGESGELSLSADYGDIIFDGNLKRTAKENAADVNGVTVSSQAISMGSGGKITTLRAKAGHQILFNDPIEMANGN
481-NQPAQSSEPLKINDGEGYTGDIVFANGNSTLYQNVTIEQGRIVLREKAKLSVNSLSQTGGSLYMEAGSTLDFVTPQPPQQ
561-PPAANQLITLSNLHLSLSSLLANNAVTNPPTNPPAQDSHPAIIGSTTAGSVTISGPIFPEDLDDTAYDRYDWLGSNQKID
641-VLKLQLGTQPSANAPSDLTLGNEMPKYGYQGSWKLAWDPNTANNGPYTLKATWTKTGYNPGPERVASLVPNSLWGSILDI
721-RSAHSAIQASVDGRSYCRGLWVSGVSNFFYHDRDALGQGYRYISGGYSLGANSYFGSSMFGLAFTEVFGRSKDYVVCRSN
801-HHACIGSVYLSTKQALCGSYLFGDAFIRASYGFGNQHMKTSYTFAEESDVRWDNNCLVGEIGVGLPIVITPSKLYLNELR
881-PFVQAEFSYADHESFTEEGDQARAFRSGHLMNLSVPVGVKFDRCSSTHPNKYSFMGAYICDAYRTISGTQTTLLSHQETW
961-TTDAFHLARHGVIVRGSMYASLTSNIEVYGHGRYEYRDTSRGYGLSAGSKVRF*

Fig. 4. Peptide mass fingerprint and identified protein sequence. Arrows indicate masses assigned to the protein sequence of PmpG. The sequence of *C. trachomatis* D PmpG is reproduced below the spectrum with identified peptides outlined in bold. Above the sequence string is indicated the partly sequenced *C. trachomatis* L2 PmpG protein (solid line indicates an identical amino acid in *C. trachomatis* L2 PmpG). Identified masses are: (Da) 988.3606 (1), 1110.4487 (2), 1173.5020 (3), 1239.4547 (4), 1255.4669 (4′), 1292.5332 (5), 1298.5652 (6), 1404.5699 (7), 1419.8324 (I), 1774.8459 (8), 1794.7871 (9), 1829.8894 (10), 1838.0288 (II), 1839.9958 (11), 1847.8554 (12), 1927.9295 (13), 1941.8916 (14), 1957.8677 (14′), 2392.2977 (15), 2480.2669 (16), 2681.3849 (17), 2697.4815 (III), 2823.4012 (18), 3218.6037 (19). Tryptic autolytic fragments are indicated by asterisks.

both the number of *pmp* genes identified, the molecular mass of predicted proteins and the primary amino acid sequence. Whereas the well studied *Chlamydia* outer membrane proteins (MOMP, Omp2 and Omp3) are more than 66% identical between species, the identified *C. trachomatis* PmpG and PmpH proteins are highly variable to homologous proteins of both *C. pneumoniae* and *C. psittaci* (sequence identities in the range of 14–30%) [17]. In both *C. pneumoniae* and *C. psittaci* a Pmp protein has been identified on the surface of EB. Perhaps the compact structure of this protein family (due to the high content of small aliphatic amino acids) or the presence of post-translational modifications makes the detection of Pmp proteins on the *Chlamydia* surface difficult by means of conventional methods using monospecific antibodies [10,18]. It has been reported that one *C. psittaci* Pmp protein (POMP90A) contains an amino-terminal exterior domain and a carboxy-terminal integral membrane portion [19]. In this respect it is interesting to observe that all repeated motifs (GGAI) are contained within the N-terminal domain in all Pmp proteins identified [10].

Analysis of the genome sequence of *C. trachomatis* serovar D showed that this strain contained a limited subset of high molecular proteins (>100 kDa) with potential outer membrane localization [2]. The original article describing the purification of COMC did show the presence of high molecular mass proteins [5]. By radioiodination, the authors reported the existence of a 105-kDa band in both *C. trachomatis* L2 and C, but not in serovar E. Similar to the PmpG/PmpH proteins identified in the present study, these proteins were not significantly stained by Coomassie blue. By ion chromatography of SDS-solubilized *C. trachomatis* L2 COMC the authors purified MOMP, but they also eluted high molecular mass proteins (>100 kDa). This is consistent with recent reports indicating that the Pmp proteins are integral outer membrane proteins [9]. Indeed, Everett and Hatch found significant labelling with a hydrophilic labelling reagent of ~90–100-kDa proteins, when analyzing *C. trachomatis* L2 COMC [18]. The detection of MOMP trimers in SDS–PAGE requires the absence of a reducing agent or chemical cross-linking of COMC [13,20]. We found no indications of trimeric MOMP in the samples analyzed by mass spectrometry. Several high molecular mass proteins were observed when analyzing the total polypeptide composition of different *C. trachomatis* serovars [21]. Surface proteins were detected by iodination and both a 118-kDa (LGV strain-specific) and a 155-kDa protein were labelled. The relation of these observations to the presence of Pmp proteins expressed in COMC is currently under investigation.

Recent reports suggest that *C. psittaci* Pmp proteins are highly immunogenic in post-abortion sheep sera [8]. Whether Pmp proteins of *C. trachomatis* are important immunogens during the progression of human infections remains to be determined. In a recent study of heterotypic immunity in murine experimental *Chlamydia* infections it was found that high levels of antibodies to one or more ~100-kDa proteins were detected when mice were infected with the *C. trachomatis* mouse pneumonitis biovar [22]. As the authors indicated, this immunogen is unknown. Identification of these immunogens may reveal the importance of Pmp proteins in naturally occurring infections, with respect to antigen variation, immunogenicity and pathogenicity. The presence of a larger repertoire of Pmp encoding genes in *C. pneumoniae* as opposed to *C. trachomatis* might suggest a putative role in the pathogenicity of *Chlamydia*, given the lack of variability in *C. pneumoniae* MOMP.

Acknowledgements

We are grateful to Kate Rafn, Karin Skovgaard Soerensen, Lisbet Wellejus Pedersen, and Inger Andersen for skillful technical assistance. P.R. is a member of the 'Center for Experimental Bioinformatics' sponsored by the Danish National Research Foundation. This work was also supported by EU Grant ERBCHRXCT920040, the Danish Health Research Council (12-1620-1), the Danish Veterinary and Agricultural Research Council (20-3503-1) and the University of Aarhus Research Foundation.

References

[1] Schachter, J. (1999) Infection and disease epidemiology. In: Chlamydia; Intracellular Biology, Pathogenesis, and Immunity (Stephens, R.S., Ed.), pp. 139–169. American Society of Microbiology, Washington, DC.

[2] Stephens, R.S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R.L., Zhao, Q., Koonin, E.V. and Davis, R.W. (1998) Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science 282, 754–759.

[3] Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R.W., Olinger, L., Grimwood, J., Davis, R.W. and Stephens, R.S. (1999) Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. Nature Genet. 21, 385–389.

[4] Struyve, M., Moons, M. and Tommassen, J. (1991) Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Mol. Biol. 218, 141–148.

[5] Caldwell, H.D., Kromhout, J. and Schachter, J. (1981) Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. 31, 1161–1176.

[6] Hatch, T.P. (1999) Developmental biology. In: Chlamydia; Intracellular Biology, Pathogenesis, and Immunity (Stephens, R.S., Ed.), pp. 29–67. American Society of Microbiology, Washington, DC.

[7] Stothard, D.R., Boguslawski, G. and Jones, R.B. (1998) Phylogenetic analysis of the *Chlamydia trachomatis* major outer membrane protein and examination of potential pathogenic determinants. Infect. Immun. 66, 3618–3625.

[8] Longbottom, D., Russell, M., Jones, G.E., Lainson, F.A. and Herring, A.J. (1996) Identification of a multigene family coding for the 90 kDa proteins of the ovine abortion subtype of *Chlamydia psittaci*. FEMS Microbiol. Lett. 142, 277–281.

[9] Longbottom, D., Findlay, J., Vretou, E. and Dunbar, S.M. (1998) Immunoelectron microscopic localisation of the OMP90 family on

the outer membrane surface of *Chlamydia psittaci*. FEMS Microbiol. Lett. 164, 111–117.
[10] Knudsen, K., Madsen, A.S., Mygind, P., Christiansen, G. and Birkelund, S. (1999) Identification of two novel genes encoding 97- to 99-kilodalton outer membrane proteins of *Chlamydia pneumoniae*. Infect. Immun. 67, 375–383.
[11] Longbottom, D., Russell, M., Dunbar, S.M., Jones, G.E. and Herring, A.J. (1998) Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the *Chlamydia psittaci* subtype that causes abortion in sheep. Infect. Immun. 66, 1317–1324.
[12] Jensen, O.N., Larsen, M.R. and Roepstorff, P. (1998) Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: Strategies and applications. Proteins Struct. Funct. Genet. 999 (Proteins Suppl. 2), 74–89.
[13] Birkelund, S., Lundemose, A.G. and Christiansen, G. (1988) Chemical crosslinking of *Chlamydia trachomatis*. Infect. Immun. 56, 654–659.
[14] Nesterenko, M.V., Tilley, M. and Upton, S.J. (1994) A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. J. Biochem. Biophys. Methods 28, 239–242.
[15] Rosenfeld, J., Capdevielle, J., Guillemot, J.C. and Ferrara, P. (1992) In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal. Biochem. 203, 173–179.
[16] Lindquist, E.A. and Stephens, R.S. (1998) Transcriptional activity of a sequence variable protein family in *Chlamydia trachomatis*. In: Chlamydial Infections: Proceedings of the Ninth International Symposium on Human Chlamydial Infection. International *Chlamydia* Symposium (Stephens, R.S. et al., Eds), pp. 259–262.
[17] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
[18] Everett, K.D.E. and Hatch, T.P. (1995) Architecture of the cell envelope of *Chlamydia psittaci* 6BC. J. Bacteriol. 177, 877–882.
[19] Buendia, A.J., Salinas, J., Sanchez, J., Gallego, M.C., Rodolakis, A. and Cuello, F. (1997) Localization by immunoelectronmicroscopy of antigens of *Chlamydia psittaci* suitable for diagnosis or vaccine development. FEMS Microbiol. Lett. 150, 113–119.
[20] Newhall, W.J. and Jones, R.B. (1983) Disulfide-linked oligomers of the major outer membrane protein of *Chlamydiae*. J. Bacteriol. 154, 998–1001.
[21] Salari, S.H. and Ward, M.E. (1981) Polypeptide composition of *Chlamydia trachomatis*. J. Gen. Microbiol. 123, 197–207.
[22] Ramsey, K.H., Cotter, T.W., Salyer, R.D., Miranpuri, G.S., Yanez, M.A., Poulsen, C.E., DeWolfe, J.L. and Byrne, G.I. (1999) Prior genital tract infection with a murine or human biovar of *Chlamydia trachomatis* protects mice against heterotypic challenge infection. Infect. Immun. 67, 3019–3025.

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Characterization of Outer Membrane Proteins in *Chlamydia trachomatis* LGV Serovar L2

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We used a photoactivatable, lipophilic reagent, 3′-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine, to label proteins in the outer membrane of elementary bodies of *Chlamydia trachomatis* LGV serovar L2 and mass spectrometry to identify the labeled proteins. The identified proteins were polymorphic outer membrane proteins E, G, and H, which were made late in the developmental cycle, the major outer membrane protein, and a mixture of 46-kDa proteins consisting of the open reading frame 623 protein and possibly a modified form of the major outer membrane protein.

The cell envelope of *Chlamydia* species resembles that of other gram-negative bacteria, consisting of an outer membrane (OM), an inner membrane (IM), and a periplasmic space. Two unusual features of the chlamydial envelope are a deficient and perhaps novel peptidoglycan structure (reviewed in references 6, 10, and 21) and the presence of a disulfide-bond-cross-linked major outer membrane protein (MOMP) in the OM and cross-linked cysteine-rich proteins (CRPs) in the periplasm (reviewed in reference 12).

The MOMP is the only chlamydial OM protein that has been well characterized. It is a porin (2, 30), is surface exposed (7, 28), and may play a role in the attachment of *Chlamydia trachomatis* to host cells (27, 28). Genes that potentially encode a family of proteins, referred to as polymorphic outer membrane proteins (Pomps), have been identified in the genomes of *Chlamydia* species: nine Pomp genes (*pmpA* to *pmpH*) are present in the genomes of *C. trachomatis* serovars D and L2, 21 genes (*pmp1* to *pmp21*) are in *C. pneumoniae*, and at least six genes (Pomp90 and Pomp98 families) are encoded by the genomes of ovine strains of *C. psittaci* (15, 20, 26). However, only two Pomps have been shown to be produced in *C. trachomatis* and *C. pneumoniae*, and only four Pomps have been identified in *C. psittaci* (11, 16, 20, 22, 29). The functions of the Pomps are not known, nor is it known when *C. trachomatis* Pomps are made during the developmental cycle. Several other OM proteins can be predicted from chlamydial genomic sequences (15, 26); however, none of these proteins have been experimentally identified in OMs.

The OM of chlamydiae is poorly characterized, in part because of the difficulty of growing large quantities of chlamydiae but mainly because chlamydial OMs cannot be separated from IMs by density gradient centrifugation. Criteria that have been used to identify chlamydial OM proteins include surface exposure, as detected by susceptibility to trypsin or reaction with antibodies following treatment of infectious elementary bodies (EBs) with these reagents, and insolubility in the weak anionic detergent sodium lauryl sarcosinate (Sarkosyl). Although these methodologies have been useful, they can yield deceptive results. For example, damage to EBs or contamination of EBs with osmotically fragile reticulate bodies during harvesting and purification can expose proteins that are not on the surface of EBs, and the failure to observe positive reactions will result if a surface-exposed protein lacks a trypsin-sensitive site or an immunodominant epitope. Insolubility in Sarkosyl is also subject to misinterpretation. The technique was originally developed by Filip et al. (9) to remove cytoplasmic membrane proteins from well-characterized integral OM proteins of *Escherichia coli* and was adapted to chlamydial studies by Caldwell et al. (4) and Hatch et al. (14). However, the reason for the differential solubility of IM and OM proteins in Sarkosyl is not known, and it is likely that some non-OM proteins fractionate in the Sarkosyl-insoluble fraction and that some OM proteins are released from OMs by Sarkosyl. For these reasons we chose to identify *C. trachomatis* LGV serovar L2 OM proteins on the basis of their reaction with 3′-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID), a photoactivatable lipophilic reagent developed by Brunner and Semenza (3) to label amino acid side chains and other ligands in the lipophilic environment of cytoplasmic membranes of eukaryotic cells. Because TID is lipophilic, it penetrates the OM of gram-negative bacteria, where it becomes trapped and incapable of passing through the hydrophilic periplasm and beyond (8).

Identification of OM proteins in *C. trachomatis* L2. EBs were harvested at 48 h after infection of L929 cells, treated with 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) to eliminate osmotically fragile reticulate bodies, and purified by centrifugation for 30 min at 80,000 × *g* on a three-step gradient of 29, 34, and 40% Hypaque-76 (Nycomed Inc., Princeton, N.J.). Purified EBs from 2×10^8 cells were reacted with 25 μCi of [^{125}I]TID, as previously described (8), and incubated for 30 min at 37°C in the presence or absence of 12 μg of trypsin (type III from bovine pancreas; Sigma Chemical Co.) in 200 μl of phosphate-buffered saline (pH 7.4), followed by the addition of 24 μg of trypsin inhibitor (type II-O from chicken egg white; Sigma). After the EBs were washed once in trypsin inhibitor, one half of the EB preparation was solubilized by heating to 90°C in Laemmli buffer (18) containing 5% β-mercaptoethanol and 10 mM dithiothreitol, and the other half was extracted with 500 μl of 0.5% Sarkosyl (Sigma) in phosphate-buffered

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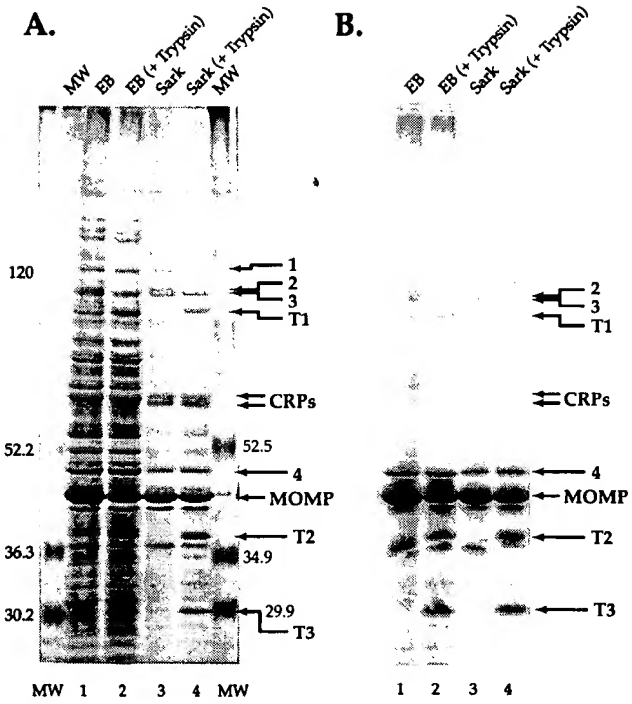

FIG. 1. Identification of proteins labeled with [^{125}I]TID by SDS-PAGE. (A) Coomassie-stained 7.5 to 15% polyacrylamide gel, with prestained protein standard sizes shown in kilodaltons at each side; (B) phosphorimage of the gel. Lanes: 1, whole EBs; 2, whole EBs following treatment with trypsin; 3, the Sarkosyl-insoluble fraction; 4, the Sarkosyl-insoluble fraction of EBs treated with trypsin. Arrows point to proteins discussed in the text.

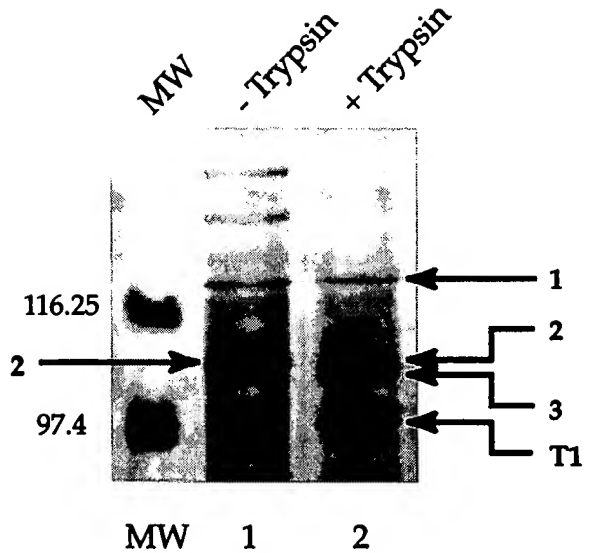

FIG. 2. Zinc-imidazole-stained 5 to 7.5% polyacrylamide gel of high-molecular-weight proteins present in the Sarkosyl-insoluble fraction of EBs. Lanes: 1, Sarkosyl-insoluble fraction of EBs; 2, Sarkosyl-insoluble fraction of EBs treated with trypsin. Sizes of prestained protein standards in kilodaltons are shown on the left. Arrows point to bands (numbered the same as in Fig. 1) that were analyzed by mass spectrometry.

saline for 30 min at 37°C. The Sarkosyl-insoluble fraction was collected by centrifugation (14,500 × *g* for 20 min) and washed twice in 500 μl of 0.5% Sarkosyl before it was solubilized in Laemmli buffer with reducing agents. Proteins in whole EBs and the Sarkosyl-insoluble fraction of EBs were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5 to15% gradient gels, and incorporation of ^{125}I into proteins was detected by phosphorimaging of the dried gels.

The protein profiles of whole EBs and the Sarkosyl-insoluble fraction of EBs are shown in Fig. 1. A number of proteins were seen in the Coomassie brilliant blue-stained Sarkosyl-insoluble fraction (Fig. 1A, lane 3), including the 40-kDa MOMP and its likely degradation products (28), the 60-kDa CRP doublet, and several unknown proteins. The unknown proteins included one of about 120 kDa (protein 1), a poorly resolved doublet of about 100 kDa (proteins 2 and 3), and a protein of approximately 46 kDa (protein 4). Only the MOMP, likely degradation products of the MOMP, and proteins 2 to 4 were labeled extensively with TID; the periplasmic CRPs, notably, were not labeled (Fig. 1B, lane 3). The TID-labeled, Sarkosyl-insoluble profile was similar to that of the TID-labeled whole EB protein profile (Fig. 1B, lanes 1 and 3). Treatment of EBs with trypsin prior to the preparation of the Sarkosyl-insoluble fraction reduced the intensity of the protein doublet 2-3, with the appearance of new peptides, designated tryptic fragments T1 to T3.

In order to better resolve the high-molecular-weight proteins, the Sarkosyl-insoluble fraction of EBs was fractionated on a 5.0 to 7.5% gel and proteins were visualized with the reversible, negative zinc-imidazole stain described by Castellanos-Serra et al. (5). Under this condition of analysis, the intensity of protein 2 was reduced when the EBs were treated with trypsin, suggesting that the band may consist of a mixture of two or more proteins (Fig. 2). Gel slices containing proteins 1 to 3 and trypsin fragment T1 were excised from the gel shown in Fig. 2 and prepared for analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry after in-gel digestion with trypsin, as described by Shevchenko et al. (25). Protein 4, the MOMP, and trypsin fragments T2 and T3 were similarly analyzed following electrophoresis of the Sarkosyl-insoluble fraction on 7.5 to 15% gels. Because the *C. trachomatis* L2 genome has not been completely sequenced and annotated, we identified proteins with the *C. trachomatis* D database located on the Protein Prospector website (http://prospector.ucsf.edu). Our findings are summarized in Table 1.

When EBs were not first treated with trypsin, protein bands 2 and 3 contained a mixture of Pomps E, G, and H. After treatment of EBs with trypsin, these bands were resolved to a mixture Pomps E and H, with PompE being the predominant protein in band 2 and PompH being the predominant protein in band 3; fragment T1 was identified as a degradation product of PompG. We concluded that the trypsin-sensitive PompG and the trypsin-insensitive PompE comigrate as band 2, with contamination with Pomp H (protein 3) due to smearing during electrophoresis. Pomps G and H were previously identified by Mygind et al. (22) in the Sarkosyl-insoluble fraction of *C. trachomatis* L2.

The best identification of band 1 was *C. trachomatis* D open reading frame (ORF) 664 (26). The predicted peptide of ORF

TABLE 1. Proteins identified in the Sarkosyl-insoluble fraction by mass spectometry[a]

Band	Identification
1	ORF 664
2 (−trypsin)	Mixture of Pomps E, G, and H
2 (+trypsin)	Mixture of Pomps E and H
3 (−trypsin)	Mixture of Pomp G and H
3 (+trypsin)	PompH
T1	PompG
4	Mixture of MOMP and ORF 623
T2	MOMP
T3	MOMP

[a] Zinc-imidazole negatively stained bands were excised from gels, and samples were prepared for MALDI-TOF mass spectrometry by in-gel digestion with trypsin as described by Shevchenko et al. (25).

664 possesses a potential FHA (forkhead-associated) domain, thought to be important in protein-protein interactions, and is weakly homologous to several proteins found in eukaryotes and prokaryotes; however, its function in chlamydiae is not clear. PSORT analysis (http://psort.nibb.ac.jp) predicted an uncleavable signal sequence and an IM location for the protein encoded by ORF 664, which is consistent with the lack of labeling of protein 1 by [^{125}I]TID. The predicted molecular weight of the mature protein encoded by ORF 664 is 89,649, considerably less than the relative molecular weight (120,000) determined by SDS-PAGE. The predicted pI of the mature protein is 4.32, and the acidic nature of the protein, including several stretches of high-density negative charge, may be responsible for its anomalous migration rate. Interestingly, ORF 664 is weakly paralogous to PompC (E value = 5e-06); however MALDI-TOF analysis failed to identify PompC of either *C. trachomatis* D or *C. trachomatis* L2 (only a partial sequence is available) in band 1.

Protein band 4 (relative molecular weight of about 46,000) was found to be a mixture of the MOMP and ORF 623 (26) by MALDI-TOF analysis, with the tryptic peptides with the highest counts being assigned to the MOMP. N-terminal amino acid analysis confirmed the presence of the MOMP and ORF 623 in protein band 4, with the MOMP being present in a ratio of about 2 to 1. ORF 623 encodes a predicted chlamydia-specific protein of unknown function; it possesses a predicted signal sequence, which was confirmed by the N-terminal sequence analysis, and the characteristics of an OM protein as determined by PSORT analysis. The predicted molecular weight of the mature form is 45,669. The presence of the MOMP in protein band 4 may be the result of smearing of the abundant 40-kDa MOMP during electrophoresis or may be due to the presence of a posttranslationally modified form of the MOMP in the band. Kuo and colleagues (17) have presented evidence that the 40-kDa MOMP of *C. trachomatis* L2 is N-linked glycosylated with a high-mannose oligosaccharide. It is possible the carbohydrate identified by Kuo et al. (17) was the result of contamination of unmodified 40-kDa MOMP with modified 46-kDa MOMP. However, in preliminary studies, we failed to detect alteration in the migration rate of any protein in the Sarkosyl-insoluble fraction when EBs were treated with calf intestinal alkaline phosphatase (Boehringer-Mannheim; 5 U in 200 μl of phosphatase buffer for 1 h at 37°C), *N*-glycosylase F (Boehringer-Mannheim; 2 U in 200 μl of phosphate-buffered saline for 1 h at room temperature, followed by the addition of Triton X-100 to 0.25% and incubation at room temperature for 30 min and then overnight at 4°C), and endoglycosylase H (Boehringer-Mannheim; 2 mU, same incubation conditions as for *N*-glycosylase F) (data not shown).

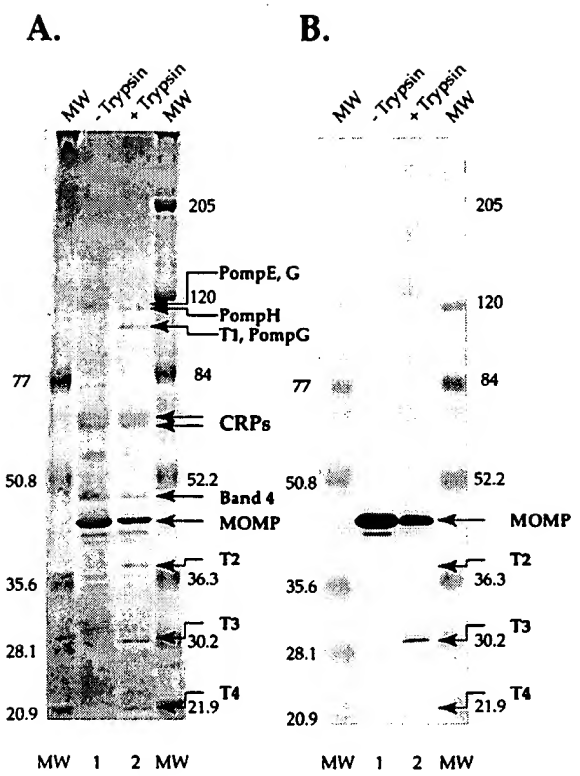

FIG. 3. SDS-PAGE (A) and immunoblot (B) analysis of the Sarkosyl-insoluble fraction of EBs incubated in the presence (lane 2) and absence (lane 1) of trypsin. The blot was treated with monoclonal antibody L2I-45, which reacts with the serovar-specific epitope in the MOMP of *C. trachomatis* L2 (1). Trypsin fragment T4 was not analyzed by mass spectrometry; however, the intensity of the band suggests that it was derived from the MOMP.

In a further attempt to identify the MOMP in band 4, an immunoblot was treated with monoclonal antibody L2I-45, which recognizes the serovar-specific epitope in the MOMP of *C. trachomatis* L2 (1). Only the abundant 40-kDa MOMP band and degradation products of the MOMP were recognized by the antibody (Fig. 3).

Stage-specific synthesis of Pomps. We recently demonstrated that three Pomps of the ovine *C. psittaci* Pomp90 family and three other potential Pomps are synthesized cotemporally with the late-stage-specific CRPs in *C. psittaci* 6BC (29). We examined stage-specific synthesis of *C. trachomatis* L2 Pomps by adding 275 μCi of [^{35}S]Cys-[^{35}S]Met (Protein Labeling Mix; NEN, Boston, Mass.) to 150-cm^2 monolayers of infected cells (2×10^7) at 14 h postinfection (p.i.) and preparing a Sarkosyl-insoluble fraction directly from the infected cells at 16, 18.5, and 23 h p.i. and from the harvested medium supernatant fluid and cells remaining attached to the flasks at 48 h p.i. Cycloheximide (0.5 μg/ml) was present during the entire infection to inhibit incorporation of label into host cells. Incorporation of label into chlamydial proteins in the Sarkosyl-insoluble frac-

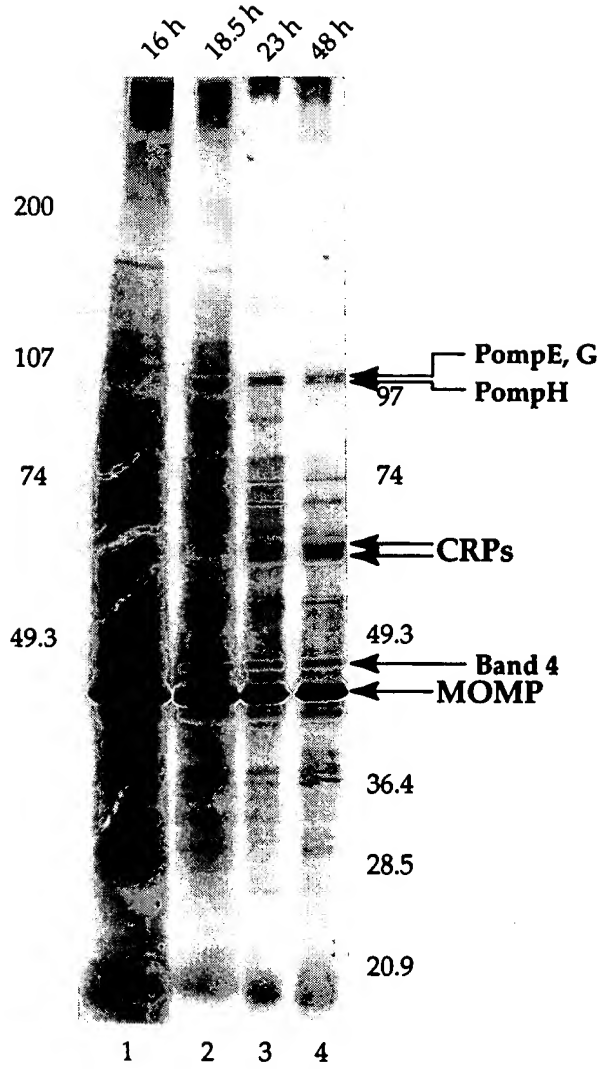

FIG. 4. Autoradiograph of a 7.5 to 15% polyacrylamide gel showing incorporation of [^{35}S]Cys-[^{35}S]Met into the Sarkosyl-insoluble fraction from 14 to 48 h p.i. The Pomps appear to be made in greater amounts late in the cycle, relative to the MOMP, but at detectable levels sooner than the late-stage-specific 60-kDa CRPs.

tion was assessed by autoradiography of a dried polyacrylamide gel, with the amount of material loaded onto the gel adjusted so that the amount of MOMP (present in relatively constant amounts at all times p.i.) for each sample was the same (Fig. 4). The results indicated that the Pomps are made at 18.5 h and later times, whereas synthesis of the late-stage-specific CRPs (13, 23) is not obvious until 23 h. The 16 h p.i. sample was difficult to interpret; the large amount of material loaded resulted in a high background and included proteins which comigrated with the CRPs that were not present at late times p.i. Nonetheless, it appears that the Pomps, at least relative to the MOMP, are not made in significant amounts between 14 and 16 h p.i.

Conclusions. [^{125}I]TID labeling of *C. trachomatis* L2 EBs identified three Pomps (E, G, and H), the MOMP, and another protein or proteins that migrate during SDS-PAGE with a relative molecular mass of about 46 kDa. It is likely that additional proteins are present in the OM but in insufficient amounts to be detected by the amount of TID label that we used. This is almost certainly the case for PompF, which is encoded immediately upstream of PompE on the same operon, with the UAA stop codon of PompF located only 2 bp upstream of the AUG start codon of PompE (26). Also, in a preliminary, study Lindquist and Stephens (19) detected transcripts of the genes encoding Pomps B, D, I, and G by reverse transcription-PCR in *C. trachomatis* L2. The Pomps that we detected are encoded by genes that are clustered in the same region of the genome: the operon encoding Pomps F and E (*pmpFE*) and three divergently transcribed genes encoding Pomps G, H, and I. It is interesting that the divergently transcribed Pomp genes are cotemporally expressed.

Mass spectrometric and N-terminal amino acid analysis indicated that the MOMP was a major constituent of the TID-labeled, 46-kDa protein band, suggesting that the protein may be a posttranscriptionally modified form of the MOMP, perhaps identical to the modified form described by Kuo et al. (17). However, the presence of the MOMP was not confirmed by immunoblot analysis and we found no evidence of glycosylated or phosphorylated protein in either the 46- or the 40-kDa protein. Alternatively, the TID-labeled protein may be encoded by ORF 623, a predicted OM protein of unknown function. Definitive identification of the proteins in the 46-kDa band will require additional investigation. Whatever the identity of the TID-labeled 46-kDa protein is, there is evidence that it may be antigenic in that Newhall et al. (24) identified a band of similar size that reacted on immunoblots of multiple serovars of *C. trachomatis* with the sera of infected patients. Although the proteins in the 46-kDa band did not possess a surface-exposed trypsin-sensitive site, their presence in the OM suggests they may be useful as diagnostic tools or in the development of a subunit vaccine.

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REFERENCES

1. **Bachr, W. B., Y.-X. Zhang, T. Joseph, S. Hua, F. E. Nano, K. D. E. Everett, and H. D. Caldwell.** 1988. Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. Proc. Natl. Acad. Sci. USA **85**:4000–4004.
2. **Bavoil, P., A. Ohlin, and J. Schachter.** 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. **44**:479–485.
3. **Brunner, J., and G. Semenza.** 1981. Selective labeling of the hydrophobic core of membranes with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iododiazirine, a carbene-generating reagent. Biochemistry **20**:7174–7182.
4. **Caldwell, H. D., J. Kromhout, and J. Schachter.** 1981. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. **31**:1161–1176.
5. **Castellanos-Serra, L., W. Proenza, V. Huerta, R. L. Moritz, and R. J. Simpson.** 1999. Proteome analysis of polyacrylamide gel-separated proteins visualized by reversible negative staining using imidazole-zinc salts. Electrophoresis **20**:732–737.
6. **Chopra, I., C. Storey, T. J. Falla, and J. H. Pearce.** 1998. Antibiotics, peptidoglycan synthesis and genomics: the chlamydial anomaly revisited. Microbiology **144**:2673–2678.
7. **Collett, B. A., W. J. Newhall, V., R. A. Jersild, Jr., and R. B. Jones.** 1989. Detection of surface-exposed epitopes on *Chlamydia trachomatis* by immune

electron microscopy. J. Gen. Microbiol. **135**:85–94.
8. **Everett, K. D. E., and T. P. Hatch.** 1995. Architecture of the cell envelope of *Chlamydia psittaci* 6BC. J. Bacteriol. **177**:877–882.
9. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. J. Bacteriol. **115**:717–722.
10. **Ghuysen, J. M., and C. Goffin.** 1999. Lack of cell wall peptidoglycan versus penicillin sensitivity: new insights into the chlamydial anomaly. Antimicrob. Agents Chemother. **43**:2339–2344.
11. **Giannikopoulou, P., K. Bini, O. D. Simitsek, V. Pallini, and E. Vretou.** 1997. Two-dimensional electrophoretic analysis of the protein family at 90 kDa of abortifacient *Chlamydia psittaci*. Electrophoresis **18**:2104–2108.
12. **Hatch, T. P.** 1996. Disulfide cross-linked envelope proteins: the functional equivalent of peptidoglycan in chlamydiae? J. Bacteriol. **178**:1–5.
13. **Hatch, T. P., M. Miceli, and J. E. Sublett.** 1986. Synthesis of disulfide-bonded outer membrane proteins during the developmental cycle of *Chlamydia psittaci* and *Chlamydia trachomatis*. J. Bacteriol. **165**:379–385.
14. **Hatch, T. P., D. W. Vance, and E. Al-Hossainy.** 1981. Identification of a major envelope protein in *Chlamydia* spp. J. Bacteriol. **146**:426–429.
15. **Kalman, S., W. Mitchell, R. Marathe, C. Lammel, J. Fan, R. W. Hyman, L. Olinger, J. Grimwood, R. W. Davis, and R. S. Stephens.** 1999. Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. Nat. Genet. **21**:385–389.
16. **Knudsen, K., A. S. Madsen, P. Mygind, G. Christiansen, and S. Birkelund.** 1999. Identification of two novel genes encoding 97- to 99-kilodalton outer membrane proteins of *Chlamydia pneumoniae*. Infect Immun. **67**:375–383.
17. **Kuo, C.-C., N. Takahashi, A. F. Swanson, Y. Ozeki, and S.-I. Hakomori.** 1996. An N-linked high-mannose type oligosaccharide, expressed at the major outer membrane protein of *Chlamydia trachomatis* mediates attachment and infectivity of the microorganism to HeLa cells. J. Clin. Investig. **98**:2813–2818.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
19. **Lindquist, E. A., and R. S. Stephens.** 1998. Transcriptional activity of a sequence variable protein family in *Chlamydia trachomatis*, p. 259–262. *In* R. S. Stephens, G. I. Byrne, G. Christiansen, I. N. Clarke, J. T. Grayston, R. G. Rank, G. L. Ridgway, P. Saikku, J. Schachter, and W. E. Stamm (ed.), Chlamydial Infections: Proceedings of the Ninth International Symposium on Human Chlamydial Infections. International Chlamydia Symposium, San Francisco, Calif.
20. **Longbottom, D., M. Russell, S. M. Dunbar, G. E. Jones, and A. J. Herring.** 1998. Molecular cloning and characterization of the genes coding for the highly immunogenic cluster of 90-kilodalton envelope proteins from the *Chlamydia psittaci* subtype that causes abortion in sheep. Infect. Immun. **66**:1317–1324.
21. **Moulder, J. W.** 1993. Why is *Chlamydia* sensitive to penicillin in the absence of peptidoglycan? Infect. Agents Dis. **2**:87–99.
22. **Mygind, P. H., G. Christiansen, P. Roepstorff, and S. Birkelund.** 2000. Membrane proteins PmpG and PmpH are major constituents of *Chlamydia trachomatis* L2 outer membrane complex. FEMS Microbiol. Lett. **186**:163–169.
23. **Newhall, W. J., V.** 1987. Biosynthesis and disulfide cross-linking of outer membrane components during the growth cycle of *Chlamydia trachomatis*. Infect. Immun. **55**:162–168.
24. **Newhall, W. J., V, B. Batteiger, and R. P. Jones.** 1982. Analysis of the human serological response to proteins of *Chlamydia trachomatis*. Infect. Immun. **38**:1181–1189.
25. **Shevchenko, A., M. Wilm, O. Vorm, and M. Mann.** 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. **68**:850–858.
26. **Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis.** 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science **282**:754–759.
27. **Su, H., and H. D. Caldwell.** 1991. In vitro neutralization of *Chlamydia trachomatis* by monovalent Fab antibody specific to MOMP. Infect. Immun. **59**:2843–2845.
28. **Su, H., Y.-X. Zhang, O. Barrera, N. G. Watkins, and H. D. Caldwell.** 1988. Differential effects of trypsin on infectivity of *Chlamydia trachomatis*: loss of infectivity requires cleavage of major outer membrane protein variable domains II and IV. Infect. Immun. **56**:2094–2100.
29. **Tanzer, R. J., D. Longbottom, and T. P. Hatch.** 2001. Identification of polymorphic outer membrane proteins of *Chlamydia psittaci* 6BC. Infect. Immun. **69**:2428–2434.
30. **Wyllie, S., R. H. Ashley, D. Longbottom, and A. J. Herring.** 1998. The major outer membrane protein of *Chlamydia psittaci* functions as a porin-like ion channel. Infect. Immun. **66**:5202–5207.

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Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of *Chlamydia trachomatis* genital tract infection: serum neutralizing IgG antibodies do not protect against chlamydial genital tract infection

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The protective efficacy of an alum-adsorbed, parenterally administered synthetic oligopeptide immunogen corresponding to antigenically common T-helper and neutralizing B-cell epitopes of the Chlamydia trachomatis *major outer membrane protein was studied in a murine model of chlamydial genital tract infection. Mice produced high levels of anti-chlamydial serum IgG neutralizing antibodies following subcutaneous immunization with the alum-adsorbed oligopeptide. Lower but detectable levels of chlamydial specific IgG antibodies were found in vaginal washes. IgG1 was the predominant isotype present in sera and vaginal washes. Chlamydial-specific IgA was not present in either the sera or vaginal washes of immunized mice. Vaccinated and control mice were challenged intravaginally or intrauterinally with low, medium, or high doses of* C. trachomatis *serovar D challenge inocula. Protection was assessed by performing quantitative chlamydial cervico-vaginal cultures over the course of the infection period. There were no statistically significant differences between groups of immunized and control mice in either colonization, shedding, or duration of infection. These findings demonstrate that parenteral immunization with the oligopeptide (serum-neutralizing antibodies) is ineffective in preventing chlamydial genital tract infection. It is possible, since chlamydial infection is restricted to the genital tract mucosae, that a more accurate evaluation of the oligopeptide vaccine potential will require local rather than systemic immunization.*

Keywords: *Chlamydia trachomatis*; oligopeptide vaccine; protective efficacy

C. trachomatis are obligate intracellular bacteria that infect epithelial cells of the oculogenital mucosae of humans causing blinding trachoma and sexually transmitted diseases (STDs)[1–3]. *C. trachomatis* serovars D–K are major causes of STDs with sequelae that include pelvic inflammatory disease (PID), ectopic pregnancy, and infertility[4–6]. Development of effective control and prevention measures is needed to decrease transmission of chlamydial genital tract infections and prevent complications of chlamydial disease. One approach towards achieving this goal is the development of an efficacious chlamydial vaccine.

We have previously described the immunogenicity of a synthetic oligopeptide, termed A8-VDIV, that corresponds to antigenically common T-helper and neutralizing B-cell epitopes of the *C. trachomatis* major outer membrane protein (MOMP)[7]. Immunization with A8-VDIV was shown to (i) generate antibodies that reacted with the targeted neutralizing LNPTIAG epitope within the VDIV sequence and which exhibited broadly cross reactive neutralizing activity against *C. trachomatis in vitro*, (ii) elicit a vigorous immune response in many congenic mouse strains differing at H-2, and (iii) effectively prime for the production of an anamnestic neutralizing antibody response following

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secondary immunization with intact chlamydiae. These immunologic characteristics of the oligopeptide are very encouraging in terms of its potential utility as a prototype chlamydial vaccine, because they show that it evokes functional heterotypic anti-chlamydial neutralizing antibodies, and primes both T and B lymphocytes that can be recalled following secondary stimulation with intact chlamydiae. Furthermore, because the oligopeptide is immunogenic in mouse strains differing at H-2, it is possible that it might be broadly recognized in genetically diverse populations such as humans.

To further delineate the vaccine potential of peptide A8-VDIV, we have studied its protective efficacy in a murine model of *C. trachomatis* genital tract infection. Our results show that mice immunized parenterally with alum-adsorbed oligopeptide produce high titered anti-chlamydial serum IgG neutralizing antibodies; however, these antibodies fail to protect mice against chlamydial genital tract infection. It is likely that a more accurate assessment of the oligopeptide vaccine's potential will require immunization protocols that are capable of evoking local rather than systemic immunity.

MATERIALS AND METHODS

Chlamydiae

C. trachomatis serovar D (UW-3/Cx) was grown in HeLa 229 cells and elementary bodies (EBs) were purified from infected cells by density gradient centrifugation as previously described[8]. A single purified preparation of serovar D EBs was prepared and used as challenge stock. The stock preparation was titered for chlamydial infectious forming units (IFUs) on monolayers of HeLa 229 cells. The 50% infectious dose (ID_{50}) of serovar D needed to infect the mouse vagina was predetermined for the experiments described below. Briefly, groups of five female A/J mice that had been progesterone treated were inoculated intravaginally with 5 μl of serial tenfold dilutions of the chlamydial stock. Infectivity was determined by performing quantitative chlamydial culture from vaginal swabs taken on days 3, 5, 7, 10 and 15 post-infection. The ID_{50} was calculated by the method of Reed and Muench[9] and was determined to be 32 IFUs (data not shown).

Immunization and sample collection

Female A/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice between 8 and 12 weeks of age were immunized subcutaneously (s.c.) with 100 μg of peptide A8-VDIV adsorbed with 0.6 mg of aluminum hydroxide gel (Rehydragel LV; Reheis Inc., Berkeley Heights, NJ) or Rehydragel LV only suspended in 0.2 ml of 0.15 M NaCl. Mice were given two booster immunizations subcutaneously with alum adsorbed peptide at 30 day intervals. Pre-bleeds and vaginal washes were collected from all mice. Mice were bled and vaginal washes collected 14 days following the last immunization. Vaginal washes were collected by refluxing a 60 μl volume of PBS containing 0.5% bovine serum albumin (PBS–BSA) twice in and out of the vaginal vault. Washes were clarified by centrifugation and kept at −20°C until used.

Measurement of chlamydial specific antibodies in serum and vaginal washes

The isotypes and levels of anti-chlamydial antibodies in sera and vaginal washes were determined by enzyme-linked immunosorbent assays (ELISA) as previously described[10]. Briefly, microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Alexandria, VA) were coated with formalin-killed serovar D EBs (10 μg ml^{-1}) in 0.05 M Tris buffer (pH 7.5) containing 0.15 M NaCl (TBS) overnight at 4°C. Antigen was removed, the plates washed with TBS containing 0.5% Tween-20 (TBS–Tween) and blocked with 2% bovine serum albumin (BSA) in TBS for 2 h at 37°C. After washing, 100 μl of serum or vaginal wash diluted with TBS–Tween containing 2% BSA were added to wells and the plates incubated at 37°C for 1 h. The plates were then washed in TBS–Tween and a 1:250 dilution of 100 μl of alkaline phosphatase labeled goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 and IgA antibodies (Southern Biotechnology Associates, Inc. Birmingham, AL) was added to the wells and incubated for 1 h at 37°C. After washing, 100 μl of substrate was added (5 mg *p*-nitrophenyl phosphate in 10 ml of 0.1 M 2:2 amino-2-methyl-1:3-propandiol, pH 10.3) to individual wells. ELISA titers are expressed as the reciprocal of serum or vaginal wash dilutions giving an absorbance reading (405 nm) of 0.3 or greater. Pooled pre-immune sera, and vaginal washes were used as negative controls and absorbance values of preimmune sera and vaginal washes were 0.1 or less. The values represent the results of duplicate determinations performed in parallel. The variation between duplicate samples was 10% or less.

Neutralization assays

In vitro *neutralization*. The *in vitro* neutralization of chlamydial infectivity was assayed on Syrian hamster kidney (HaK) cells cultivated in 96-well flat-bottom plates as previously described[11].

In vivo *neutralization*. Pooled sera from vaccinated and control mice were heat inactivated (56°C, 30 min) and diluted 1:20 in 10 mM sodium phosphate, pH 7.2, containing 0.25 M sucrose and 5 mM L-glutamic acid (SPG). A 50 μl volume of sera was incubated with a 50 μl suspension of serovar D EBs containing 6×10^5, 6×10^6 or 6×10^7 IFUs. The final dilution of sera incubated with chlamydiae was 1:40. The suspensions were incubated at 37°C for 30 min. A 5 μl volume of the suspensions, corresponding to 10^2, 10^3, and 10^4 ID_{50}, was then inoculated intravaginally into groups of 6–7 mice each. The *in vivo* neutralizing activities of the sera were assessed by culturing individual mice for chlamydiae at day 3 post-inoculation.

Experimental design of vaccine studies

Female A/J mice (8–12 weeks of age) were divided into four experimental groups. Two groups were vaccinated with alum adsorbed peptide as described above and two groups were immunized with alum only and served as controls. All mice were given 2.5 mg progesterone (Depo-Provera, UpJohn, Kalamazo, MI) s.c. 10 and 3 days prior to infectious challenge. Each of these four groups were further divided into three groups,

which were then subsequently challenged either intravaginally or intrauterinally with the three different doses of chlamydial challenge inocula. The challenge doses were 10, 10^2, and 10^3 ID_{50} which correspond to 3×10^2, 3×10^3, and 3×10^4 IFUs, respectively. The groups are designated below as intravaginal or intrauterine challenge groups, vaccinated and controls, with each of these groups subdivided into the three different ID_{50} challenge groups. Mice were challenged and cultured for chlamydiae as described below. In addition, 2–4 animals from each group were sacrificed at 14–21 days post-challenge and their genital tracts were collected for clinical evaluation and histopathology.

Challenge studies

Vaginal challenge. Vaccinated and control mice (9–10 per group) were inoculated intravaginally with 5 μl of a suspension of *C. trachomatis* serovar D in SPG containing 10, 10^2, and 10^3 ID_{50} of serovar D.

Uterine horn challenge. Five to seven mice were used for each experimental group. Anesthetized mice were placed in dorsal recumbency and the ventral abdomen prepped for surgery. A small (5–10 mm long) midline incision was made through the skin, abdominal wall and peritoneum, approximately 10 mm proximal to the pubis. Using a blunt probe, the cervix and uterine body were gently retracted and exposed through the incision site. The uterine horns were visualized, supported with the probe, and injected intraluminally using a 27 gauge needle with 10 μl of a suspension of serovar D containing 10, 10^2, and 10^3 ID_{50}. Following inoculation, the uterus was returned to a normal position and all three incisional layers were simultaneously closed using a single 9 mm wound clip. All surgery was performed utilizing appropriate aseptic sterile technique.

Chlamydial culture

Chlamydial colonization, shedding, and resolution of infection in each challenge group was assessed by quantifying the number of IFUs recovered from cervicovaginal swabs taken at 3, 8, 12, 15, 18, 22, 25 and 28 days for the intravaginal challenge groups and at 3, 5, 7, 10 and 12 days for the intrauterine groups. A calcium alginate swab (Calgiswab, type 1, Spectrum laboratories, Inc., Houston, TX) was inserted into the vagina and rotated 10 times. The swab was then placed into a 2.0 ml sterile screw cap microfuge tube containing 0.5 ml of SPG and two 5 mm sterile glass beads. The microfuge tube was vortexed for 2 min at room temperature. A 50 μl volume of the suspension was inoculated in triplicate onto HeLa 229 cell monolayers grown in 96 well plates. The plates were centrifuged at 2000 revs min^{-1} for 1 h at 24°C and then incubated at 37°C for 30 min. The inoculum was removed and the cells washed three times with 100 μl of Hank's balanced salt solution (HBSS). Monolayers were re-fed with 200 μl of MEM supplemented with 10% fetal bovine sera and 2 μg ml^{-1} cycloheximide and incubated at 37°C with 5% CO_2 for 48 h. The monolayers were washed with PBS, fixed with absolute methanol, and stained by indirect immunofluorescence using a genus-specific monoclonal antibody (EVI-HI) against chlamydial lipopolysaccharide. The number of chlamydial infectious forming units (IFUs) in three 100 × microscopic fields was determined and these values were used to compute the total number of IFUs present in a given specimen.

Clinical evaluation and histopathology

Following euthanasia, a midline incision from the pubis to the sternum provided visual access of the genital tract and allowed gross pathological evaluation. The entire genital tract was removed as a single unit, placed between two histo-sponges, snapped into a standard tissue cassette and place in 10% buffered formalin. After fixation, the genital tracts were sectioned longitudinally and the sections stained with hematoxylin–eosin. The vagina, cervix, uterus, oviduct and ovaries were individually evaluated for type (acute, subacute, chronic) and degree (minimal, moderate, and marked) of inflammation and related histopathological changes.

RESULTS

Isotype of chlamydial specific antibodies in sera and vaginal washes following immunization with peptide A8-VDIV

Pooled sera and vaginal washes from individual mice of each challenge group (intravaginal and intrauterine; vaccinated and controls) were tested by ELISA to determine both the isotype and levels of chlamydial specific antibodies prior to infectious challenge (*Figure 1*). The total IgG serum ELISA antibody titers against serovar D EBs were similar for all experimental groups, ranging from 1:8192 to 1:32 768. The predominant anti-chlamydial antibody isotype was IgG1. Serum IgG2b was present in pooled sera in all experimental groups and IgG2a was present in four of the six experimental groups. Chlamydial specific IgA was not detected in the sera of any of the vaccinated mice. Levels of IgG antibodies in vaginal washes were considerably lower, ranging in titer from 1:32 to 1:256. In general, the titers and profiles of the antibody isotypes present in vaginal washes paralleled those found in sera indicating that the source of IgG in washes was a result of serum transudation. Chlamydial specific IgA was not detectable in the vaginal washes of any of the vaccinated mice. These findings show that peptide A8-VDIV adsorbed to alum is capable of eliciting an excellent anti-chlamydial antibody response. Moreover, the finding that the predominant IgG isotype was IgG1 is suggestive that immunization with the peptide stimulated primarily a Th2-type dependent response[12–14].

ELISA IgG antibody titers in the sera and vaginal washes of individual mice immunized with peptide A8-VDIV

The chlamydial specific ELISA IgG antibody titers in the serum and vaginal washes of individual mice within each of the challenge groups are shown in *Figure 2*. All mice produced significant levels of serum IgG antibodies reactive against serovar D EBs ranging from 1:512 to 1:32 768, with a mean titer of 1:8192. IgG antibody titers in vaginal washes of mice were considerably lower than those found in sera, ranging for 1:8 to 1:512, with a mean titer of 1:64. Mice whose serum antibody titers

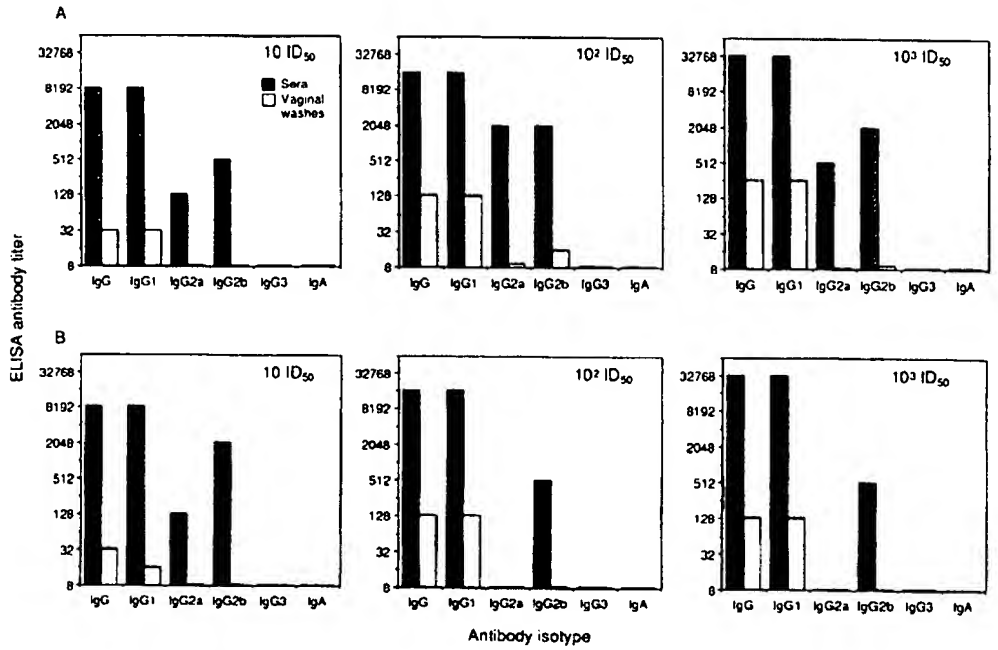

Figure 1 ELISA analysis of the isotype and titer of chlamydial specific antibodies in the sera and vaginal washes of vaccinated mice. (A) Intravaginal challenge groups (10, 10^2, and 10^3 ID_{50}) and (B) intrauterine challenge groups (10, 10^2, and 10^3 ID_{50}). Sera and vaginal washes of mice within each group were pooled and assayed by ELISA for the isotype and titer of chlamydial specific antibodies. Note that the relative titers of chlamydial specific IgG in pooled sera and vaginal washes among mice were similar. The predominant isotype was IgG1. Chlamydial specific IgA was not detected in either the sera or vaginal washes of A8-VDIV immunized mice

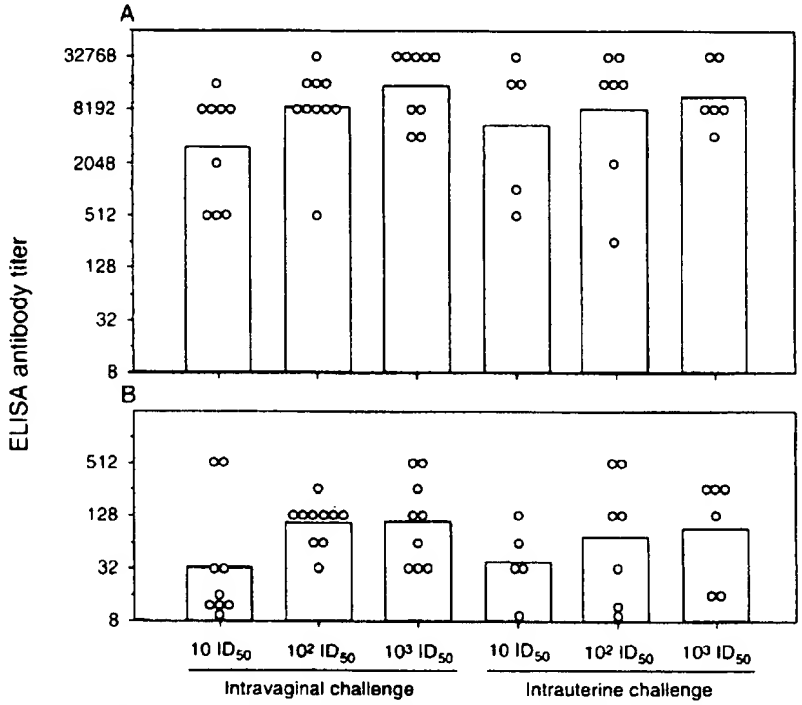

Figure 2 ELISA anti-chlamydial antibody titers in the sera and vaginal washes of A8-VDIV immunized mice. (A) Serum anti-chlamydial antibody titers against serovar D. (B) Vaginal wash anti-chlamydial antibody titers against serovar D. The IgG antibody titers against serovar D in sera and vaginal washes for individual mice are shown. Bars represent geometric mean antibody titers for mice in each of the experimental groups

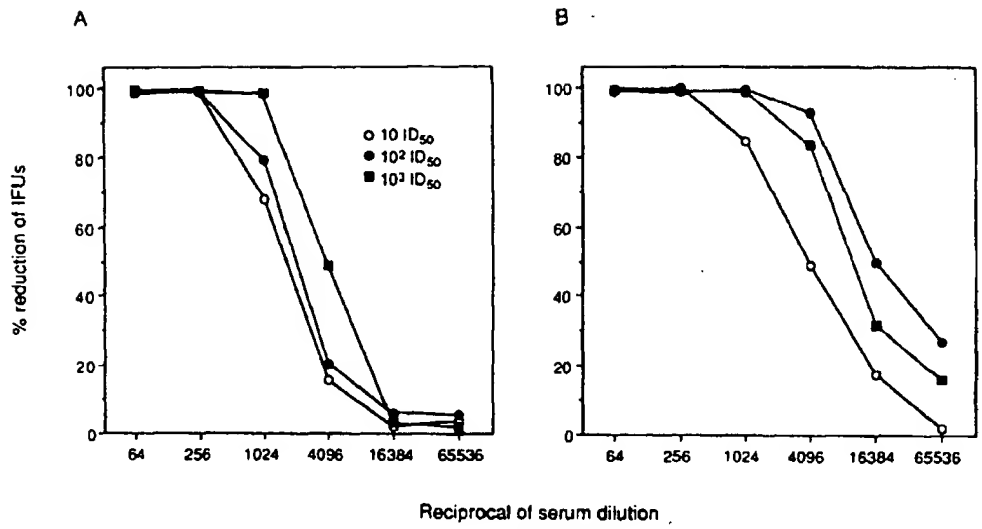

Figure 3 *In vitro* neutralization of *C. trachomatis* infectivity by mouse anti-A8-VDIV sera. (A) Serum-neutralizing titers against serovar D of the vaginal challenge groups. (B) Serum-neutralizing titers against serovar D of the uterine horn challenge groups. The serum anti-serovar D neutralizing activity was comparable for each of the experimental groups with 50% endpoint titers ranging from 1:20 48 to 1:8192

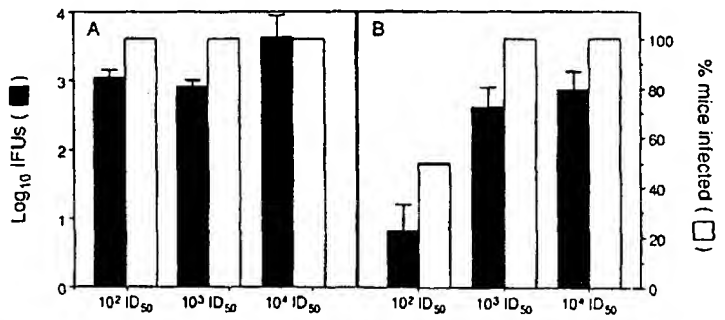

Figure 4 *In vivo* neutralization of *C. trachomatis* infectivity by mouse anti-A8-VDIV sera. (A) *In vivo* neutralizing activity of pooled sera from control mice. (B) *In vivo* neutrallzing activity of pooled sera from A8-VDIV vaccinated mice. Sera were heat-inactivated and incubated with 10^2, 10^3, or 10^4 ID_{50} of *C. trachomatis* serovar D. The serum and chlamydial suspensions were then inoculated intravaginally into groups of 6–7 mice each. Neutralization was assessed by chlamydial culture at day 3 post-challenge and determining the number of mice infected and the total number of recoverable IFUs. The final serum dilution in each of neutralization assays was 1:40. The numbers of IFUs recovered from mice inoculated with immune sera incubated with 10^2 ID_{50} were significantly lower ($P<0.005$, two-tailed Student's *t*-test) as compared with control sera incubate with the same chlamydial ID_{50}

were less than 1:512 did not have detectable levels of chlamydial specific IgG antibodies in their vaginal washes.

In vitro neutralizing antibody titers of sera from vaccinated mice

To assess whether the anti-chlamydial serum IgG antibodies were functional, pooled sera from each experimental group were assayed for their ability to neutralize the infectivity of serovar D for HaK cells (*Figure 3*). The sera of mice in the vaginal and uterine ID_{50} challenge groups all showed significant anti-chlamydial neutralizing activity with neutralizing 50% endpoints (ND_{50}) ranging between 1:2046 (intravaginal 10 ID_{50} group) to 1:16 384 (intrauterine 100 ID_{50} group). These findings show that immunization with the oligopeptide generates a vigorous and consistent anti-chlamydial neutralizing antibody response.

In vivo neutralizing activity of sera from vaccinated mice

It was important to determine if the serum antibodies produced following immunization with the oligopeptide were capable of neutralizing chlamydial infectivity for mouse vaginal epithelial cells, since these cells were the targets for infection in subsequent challenge studies. To assess *in vivo* neutralizing activity, pooled sera from vaccinated and control mice were incubated with serovar D and the mixtures inoculated intravaginally into mice. Neutralization was assessed by chlamydial culture at three days post-challenge (*Figure 4*). The sera from vaccinated mice incubated with 10^2 ID_{50} serovar D EBs (3×10^3 IFUs) showed significant neutralizing activity following intravaginal challenge. Mice inoculated with EBs pre-treated with sera from vaccinated animals showed a 50% reduction in infection rate (colonization) compared to controls. There was also a significant difference in the numbers of chlamydiae shed from the

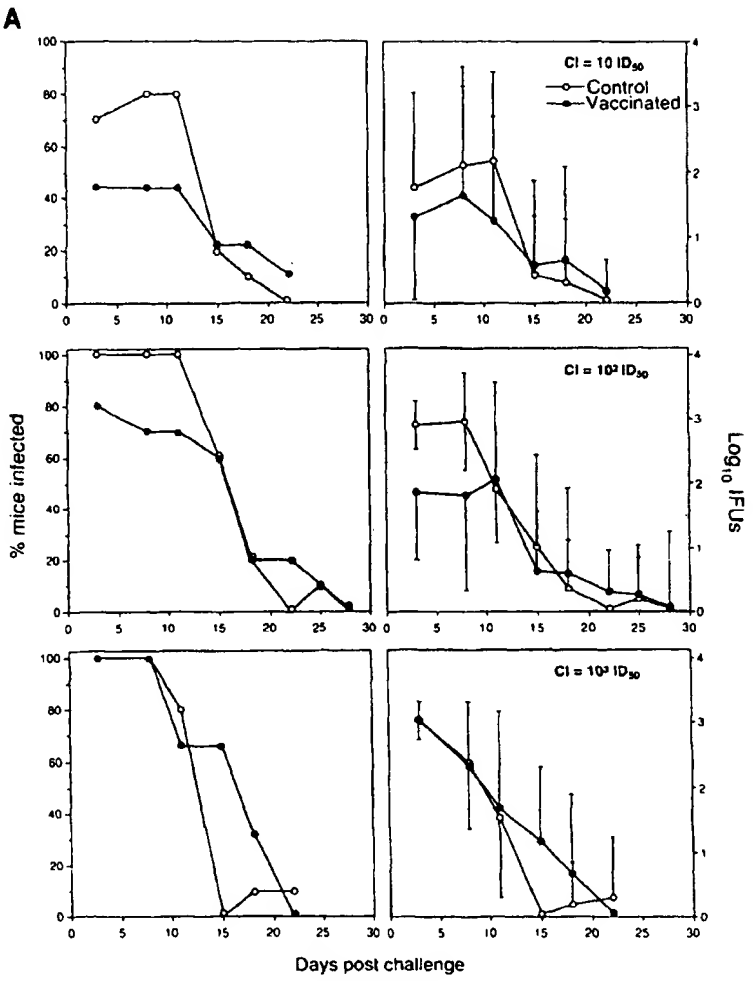

Figure 5(A)

vagina of the infected mice in this group compared to controls (0.8 vs 3.04 $\log_{10}$, respectively). This *in vivo* neutralizing activity was overcome when the concentration of chlamydiae used in the assay was increased tenfold. This is not particularly surprising, however, since only a small number of non-neutralized chlamydiae (less than 100 IFUs) are capable of establishing an infection of the mouse vagina. These findings demonstrate that the serum IgG antibodies generated following immunization with the oligopeptide are capable of neutralizing the infectivity of chlamydiae for vaginal epithelial cells the target cells of chlamydial infection in subsequent challenge studies.

Challenge experiments

Intravaginal challenge. Vaccinated and control mice were challenged with 10, 10^2, or 10^3 ID_{50} intravaginally. Protective efficacy was determined by performing quantitative chlamydial culture from cervico-vaginal swabs taken at different time periods following infectious challenge (*Figure 5A*). The percentage of animals infected following challenge with 10 and 10^2 ID_{50} was less in the vaccinated mice compared to unvaccinated controls (left hand panel). In the 10 ID_{50} challenge group, 80% of the controls were infected compared to a 50% infection rate in vaccinated mice. Similarly, the incidence of infection in control animals challenged with 10^2 ID_{50} was 100% compared to an 80% infection rate in vaccinated mice. There was no difference in the incidence of infection between vaccinated and control mice challenged with 10^3 ID_{50}. The right-hand panel in the figure shows chlamydial culture results obtained from vaccinated and control mice over the entire infection period. Less IFUs (0.5–1.0 $\log_{10}$) were recovered from vaccinated mice at days 3 and 8 post-challenge in the low and medium challenge groups. Differences in infection rates and shedding in the low and medium dose challenge groups were not statistically significant between the vaccinated and control groups, but there was a trend indicating that lower incidences of infection and recoverable IFUs correlated with lower infectious challenge doses. These results show that serum anti-chlamydial neutralizing antibodies are largely ineffective in preventing chlamydial colonization of the vaginal epithelia. Moreover, the results indicate that serum IgG antibodies do not

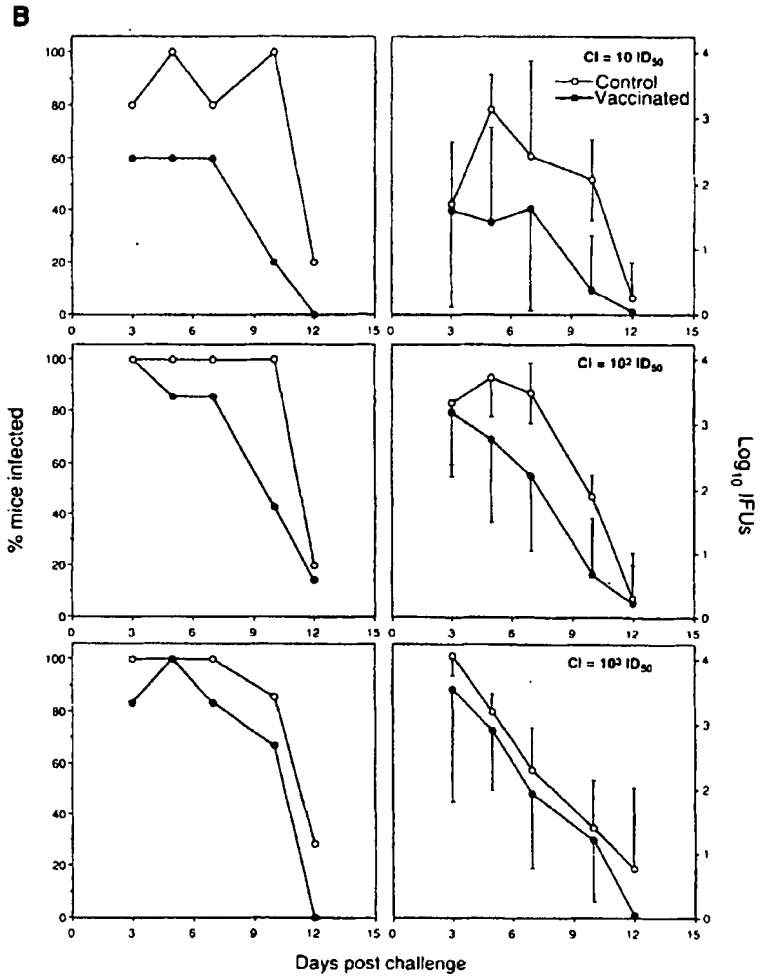

Figure 5(B)

Figure 5 Protective efficacy of A8-VDIV vaccinated mice to intravaginal and intrauterine chlamydial challenge. Infection rate and re-isolation of chlamydiae from vaginally (A) and uterine horn (B) challenged mice vaccinated with peptide A8-VDIV and alum adjuvant alone. Vaccinated and control mice were challenged vaginally or into the lumen of the uterine horns with 10, 10^2, and 10^3 ID_{50} of *C. trachomatis* serovar D. Chlamydial IFUs are expressed as the mean μ the standard deviation of 5–10 animals per experimental group. The paired mean values at each time point and at each challenge dose were not significantly different from each other as judged by examination of the 95% confidence intervals associated with each mean. The large variation in the mean values of recoverable IFUs is due in large part to the inclusion of culture negative animals (IFU=0) in the analyses

have a significant effect on either the numbers of infectious chlamydiae shed from the cervico-vagina or the resolution of chlamydial genital tract infection.

Intrauterine challenge. Mice infected vaginally with *C. trachomatis* serovar D failed to develop ascending infection of the genital tract (data not shown). Therefore, to evaluate the protective effect of serum-neutralizing antibodies on chlamydial upper genital tract infection, the uterine horns of mice were injected intraluminally with 10, 10^2 and 10^3 ID_{50} and protection was assessed by cervico-vaginal culture (*Figure 5B*). In general, the findings of these studies were very similar to those described above for the intravaginally challenged mice. There were differences in both the infection rate and numbers of chlamydiae shed at early culture periods for A8-VDIV immunized mice in the low and medium dose challenge groups; however, these differences were not statistically significant.

Histopathological profiles of the genital tract of mice challenged either intravaginally or intrauterinally with *C. trachomatis* serovar D at each of three challenge doses showed no significant inflammatory infiltrate. It was therefore not possible in these studies to evaluate the protective effect of serum anti-chlamydial IgG neutralizing antibodies on chlamydial genital tract disease.

DISCUSSION

The MOMP of *C. trachomatis* is the primary target of chlamydial neutralizing antibodies[15–19] and is considered to be the most promising candidate for the

development of a subunit chlamydial vaccine. MOMP exhibits extensive antigenic variation among different *C. trachomatis* serovars and immunodominant neutralizing responses are commonly directed to serovar-specific antigenic determinants of this protein[20–22]. In attempts to design a synthetic peptide vaccine capable of conferring a broadly cross-neutralizing antibody response against chlamydiae, we synthesized a peptide immunogen corresponding to antigenically common Th and B-cell epitopes of the MOMP[7]. We showed previously that the oligopeptide A8-VDIV was an excellent immunogen, being capable of generating high titered antibodies that neutralized the *in vitro* infectivity of those *C. trachomatis* serovars most commonly associated with chlamydial STDs. In the presently described study we took the next step to evaluate the vaccine potential of peptide A8-VDIV by studying its protective efficacy in a murine model of *C. trachomatis* genital tract infection. We found that peptide A8-VDIV, when adsorbed to alum and administered parenterally, elicited high titered serum anti-chlamydial IgG neutralizing antibodies. However, parenteral vaccination with the peptide was not effective in preventing *C. trachomatis* genital tract infection, despite moderate levels of serum chlamydial specific IgG antibodies being present in secretions of the genital mucosa. These results are not particularly surprising for several reasons. First, the most important antibody isotype in mucosal secretions that functions to provide protective immunity at mucosal surfaces is IgA[23–25]. Secondly, B-cells in mucosal effector sites of the lamina propria secret polymeric IgA (pIgA), which unlike IgG, is selectively transcytosed across the epithelium into external mucosal secretions by a secretory component (sc) dependent mechanism[26–28]. When pIgA acquires sc it becomes highly resistant to proteolytic cleavage[29] and, therefore, is able to sustain effector antibody functions in the protease rich milieu of mucosal secretions. *C. trachomatis* infection is restricted to the genital tract mucosa; therefore, it is not unreasonable to surmise that sIgA would provide a more formidable barrier than IgG in preventing chlamydial colonization and infection within this environment. Clearly, sIgA is likely to represent the first line of defence against chlamydial infection of genital tract mucosa and would therefore be expected to be more closely associated with protection than serum antibodies. In humans there is indirect evidence to support this reasoning. Brunham *et al.*[30] showed a striking correlation between the titers of chlamydial specific sIgA antibodies in cervical secretions and reduced levels of chlamydial shedding from the cervix. In contrast, there was no correlation between the levels of local or serum anti-chlamydial IgG antibodies and reduced chlamydial shedding. These findings strongly support a role for local sIgA in protection against chlamydial infection in humans, which is also likely to be the case in chlamydial infection of the murine genital tract. Our findings show that mice vaccinated parenterally with peptide A8-VDIV do not produce either serum or local anti-chlamydial IgA antibodies. The inability of parenteral immunization with the oligopeptide to evoke local (vaginal) neutralizing antibodies is a likely explanation for its ineffectiveness. A more accurate assessment of the vaccine potential of peptide A8-VDIV will depend on achieving immunization that can evoke and sustain an anti-chlamydial sIgA response at the genital tract mucosa. We are attempting to achieve this goal by chemically conjugating peptide A8-VDIV to the B subunit of cholera toxin.

Our findings clearly demonstrate that the A8-VDIV oligopeptide is immunogenic when administered in an alum-based adjuvant. Mice immunized with the peptide and challenged with chlamydiae exhibited no gross pathological or histopathological evidence suggestive of vaccine-induced hypersensitization. These properties indicate that phase I clinical trials with the peptide vaccine could be done safely in humans, which might be worthy of consideration, particularly if future studies using relevant animal models of chlamydial disease demonstrate a protective role for mucosal or serum-neutralizing antibodies.

Several other features of the immunogenicity of peptide A8-VDIV adsorbed with alum are relevant to its potential utility as a first generation chlamydial vaccine and deserve comment. The ability of alum adsorbed peptide A8-VDIV to generate a chlamydial specific IgG antibody response strongly supports the conclusion that the Th-cell epitopes contained within the peptides sequence are functional. Secondly, the finding that IgG1 was the predominant chlamydial specific antibody produced following immunization suggests that Th2 cells[12–14] were the primary responding T-cell subset stimulated by the oligopeptide. This observation may be important in terms of future studies focused on mucosal immunization with the oligopeptide, since it is known that Th2-type T-cells predominate in mucosal tissues and secrete high levels of IL-5 which induces isotype switching to B-cells producing IgA[31].

Similar work to that described here has been reported by Tuffrey *et al.*[32] using a recombinant fragment of serovar L1 MOMP as an immunogen. They found that parenteral immunization with purified recombinant L1 MOMP fragment did not reduce chlamydial colonization of the lower genital tract, but did reduce the proportion of mice developing severe salpingitis. Thus, the effect of serum anti-chlamydial neutralizing antibodies on colonization and infection of the mouse genital tract is a consistent finding in our and their studies. Unlike their results, however, we did not observe significant pathological changes in the mouse genital tract following *C. trachomatis* infection, and therefore could not access the effects of parenteral immunization on disease outcome. It is not clear why our studies failed to produce significant pathological changes in the mouse model; however, several experimental parameters could contribute to these results. First, different mouse strains were used. Second, the strain of chlamydiae used in our studies had been extensively cultivated *in vitro*, whereas the strains employed by Tuffrey *et al.* had undergone less *in vitro* passages subsequent to their isolation from humans which might conceivably affect their pathogenicity for mice. None the less, it is apparent from the work reported here and by Tuffrey *et al.* that parenteral immunization capable of eliciting systemic anti-chlamydial neutralizing antibodies alone is not likely to be an effective vaccine strategy for the prevention of chlamydial genital tract infection.

A major shortcoming of the studies described here was the inability to access the effect of systemic anti-chlamydial neutralizing antibodies on chlamydial genital

tract disease. This is particularly relevant in terms of upper genital tract infection and disease, since pathological changes at these anatomical sites in humans, e.g. salpingitis and its sequelae, can result in PID, ectopic pregnancy, and infertility. Although not ideal, a chlamydial vaccine capable of preventing or significantly reducing these complications would be useful and could serve as a first generation vaccine against chlamydial STDs. Because we consider this a worthy goal, it is our opinion that future vaccine efficacy studies employing the mouse as a model should be done using the murine *C. trachomatis* mouse pneumonitis (MoPn) strain and not human *C. trachomatis* strains. Unlike human *C. trachomatis* strains, the MoPn strain produces ascending infection of the mouse genital tract and evokes a marked acute inflammatory response of the sub-mucosal tissue. Ascending infection of the upper genital tract results in hydrosalpinx formation and infertility[33–35] sequelae that closely parallel those observed following chlamydial infection of the human genital tract. Thus, by using the murine strain, vaccine efficacy could be evaluated both in terms of chlamyidal infection and disease of the genital tract. Furthermore, the MOMP of the MoPn strain shares considerable homology with the MOMPs of human *C. trachomatis* strains[36,37]. Therefore, subunit or recombinant-based MOMP immunogens derived from the MoPn strain that might prove to be efficacious in the murine model, could be used to design similar vaccine candidates against human *C. trachomatis* biovars, and their protective efficacy tested in humans or non-human primate animal models of *C. trachomatis* infection.

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REFERENCES

1 Grayston, J.T. and Wang, S.-P. New knowledge of chlamydiae and the diseases they cause. *J. Infect. Dis.* 1975, **132**, 87–104
2 Schachter, J. Chlamydial infections (third of three parts). *N. Engl. J. Med.* 1978, **298**, 540–548
3 Schachter, J. The intracellular life of *Chlamydia*. *Curr. Top. Microbiol. Immun.* 1988, **138**, 109–139
4 Fraiz, J. and Jones, R.B. Chlamydial infections. *Annu. Rev. Med.* 1988, **39**, 357–370
5 Kuo, C.-C., Wang, S.-P., Holmes, K.K. and Grayston, J.T. Immunotypes of *Chlamydia trachomatis* isolates in Seattle, Washington. *Infect. Immun.* 1983, **41**, 865–868
6 Morrison, R.P., Manning, D.S. and Caldwell, H.D. Immunology of *Chlamydia trachomatis* infections: immunoprotective and immunopathogenetic responses. In: *Advances in Host Defense Mechanisms* (Ed. Quinn, T.C.). Raven Press, Ltd, New York, 1992, pp. 57–84
7 Su, H. and Caldwell, H.D. Immunogenicity of a synthetic oligopeptide corresponding to antigenically common T-helper and B-cell neutralizing epitopes of the major outer membrane protein of *Chlamydia trachomatis*. *Vaccine* 1993, **11**, 1159–1166
8 Caldwell, H.D., Kromhout, J. and Schachter, J. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect. Immun.* 1981, **31**, 1161–1176
9 Reed, L.J. and Muench, H. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* 1938, **27**, 493–497
10 Su, H., Morrison, R.P., Watkins, N.G. and Caldwell, H.D. Identification and characterization of T helper cell epitopes of the major outer membrane protein of *Chlamydia trachomatis*. *J. Exp. Med.* 1990, **172**, 203–212
11 Su, H. and Caldwell, H.D. *In vitro* neutralization of *Chlamydia trachomatis* by monovalent Fab antibody specific to the major outer membrane protein. *Infect. Immun.* 1991, **59**, 2843–2845
12 Munoz, E., Zubiaga, A.M., Merrow, M., Sauter, N.P. and Huber, B.T. Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation. *J. Exp. Med.* 1990, **172**, 95–103
13 Snapper, C.M. and Paul, W.E. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 1987, **236**, 944–947
14 Finkelman, F.D., Holmes, J., Katona, I.M. *et al.* Lymphokine control of *in vivo* immunoglobulin isotype selection. *Annu. Rev. Immun.* 1990, **8**, 303–333
15 Lucero, M.E. and Kuo, C.-C. Neutralization of *Chlamydia trachomatis* cell culture infection by serovar-specific monoclonal antibodies. *Infect. Immun.* 1985, **50**, 595–597
16 Peeling, R., Maclean, I.W. and Brunham, R.C. *In vitro* neutralization of *Chlamydia trachomatis* with monoclonal antibody to an epitope on the major outer membrane protein. *Infect. Immun.* 1984, **46**, 484–488
17 Su, H., Watkins, N.G., Zhang, Y.-X. and Caldwell, H.D. *Chlamydia trachomatis*–host cell interactions: role of the chlamydial major outer membrane protein as an adhesin. *Infect. Immun.* 1990, **58**, 1017–1025
18 Zhang, Y.-X., Stewart, S., Joseph, T., Taylor, H.R. and Caldwell, H.D. Protective monoclonal antibodies recognize epitopes located on the major outer membrane protein of *Chlamydia trachomatis*. *J. Immun.* 1987, **138**, 575–581
19 Zhang, Y.-X., Stewart, S.J. and Caldwell, H.D. Protective monoclonal antibodies to *Chlamydia trachomatis* serovar-and serogroup-specific major outer membrane protein determinants. *Infect. Immun.* 1989, **57**, 636–638
20 Baehr, W., Zhang, Y.-X., Joseph, T. *et al.* Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. *Proc. Natl Acad. Sci. USA* 1988, **85**, 4000–4004
21 Conlan, J.W., Clarke, I.N. and Ward, M.E. Epitope mapping with solid-phase peptides: identification of type-, subspecies-, species- and genus-reactive antibody binding domains on the major outer membrane protein of *Chlamydia trachomatis*. *Molec. Microbiol.* 1988, **2**, 673–679
22 Stephens, R.S., Wagar, E.A. and Schoolnik, G.K. High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of *Chlamydia trachomatis*. *J. Exp. Med.* 1988, **167**, 817–831
23 Underdown, B.J. and Schiff, J.M. Immunoglobulin A: strategic defense initiative at the mucosal surface. *Annu. Rev. Immun.* 1986, **4**, 389–417
24 Kiyono, H., Bienenstock, J., McGhee, J.R. and Ernst, P.B. The mucosal immune system: features of inductive and effector sites to consider in mucosal immunization and vaccine development. *Reg. Immun.* 1992, **4**, 54–62
25 Kraehenbuhl, J.-P. and Neutra, M.R. Molecular and cellular basis of immune protection of mucosal surfaces. *Physiol. Rev.* 1992, **72**, 853–879
26 Mestecky, J. and McGhee, J.R. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune responses. *Adv. Immun.* 1987, **40**, 153–245
27 Mostov, K.E. Transepithelial transport of immunoglobulins. *Annu. Rev. Immun.* 1994, **12**, 63–84
28 Lamm, M.E. Cellular aspects of immunoglobulin A. *Adv. Immun.* 1976, **22**, 223–290
29 Underdown, B.J. and Dorrington, K.J. Studies on the structural and conformational basis for the relative resistance of serum and secretory immunoglobulin A to proteolysis. *J. Immun.* 1974, **112**, 949–959
30 Brunham, R.C., Kuo, C.-C., Cles, L. and Holmes, K.K. Correlation of host immune response with quantitative recovery of *Chlamydia trachomatis* from the human endocervix. *Infect. Immun.* 1983, **39**, 1491–1494
31 Harriman, G.R. and Strober, W. Interleukin 5, a mucosal lymphokine. *J. Immun.* 1987, **139**, 3553–3555
32 Tuffrey, M., Alexander, F., Conlan, W., Woods, C. and Ward, M. Heterotypic protection of mice against chlamydial salpingitis and colonization of the lower genital tract with a human serovar

F isolate of *Chlamydia trachomatis* by prior immunization with recombinant serovar L1 major outer-membrane protein. *J. Gen. Microbiol.* 1992, **138**, 1707–1715

33 Barron, A.L., White, H.J., Rank, R.G., Soloff, B.L. and Moses, E.B. A new animal model for the study of *Chlamydia trachomatis* genital infections: infection of mice with the agent of mouse pneumonitis. *J. Infect. Dis.* 1981, **143**, 63–66

34 Patton, D.L., Landers, D.V. and Schachter, J. Experimental *Chlamydia trachomatis* salpingitis in mice: initial studies on the characterization of the leukocyte response to chlamydial infection. *J. Infect. Dis.* 1989, **159**, 1105–1110

35 Swenson, C.E., Donegan, E. and Schachter, J. *Chlamydia trachomatis*-induced salpingitis in mice. *J. Infect. Dis.* 1983, **148**, 1101–1107

36 Stephens, R.S. and Kuo, C.-C. *Chlamydia trachomatis* species-specific epitope detected on mouse biovar outer membrane protein. *Infect. Immun.* 1984, **45**, 790–791

37 Fielder, T.J., Pal, S., Peterson, E.M. and de la Maza, L.M. Sequence of the gene encoding the major outer membrane protein of the mouse penumonitis biovar of *Chlamydia trachomatis*. *Gene* 1991, **106**, 137–138

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Partial protection against genital reinfection by immunization of guinea-pigs with isolated outer-membrane proteins of the chlamydial agent of guinea-pig inclusion conjunctivitis

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Because partial protection against reinfection is induced by experimental infection in the guinea-pig model of genital chlamydial infection, we sought to induce immunity by immunization. Female guinea-pigs were immunized subcutaneously with the major outer-membrane protein (MOMP) and the 61 kDa cysteine-rich outer-membrane protein (61 kDa) of the agent of guinea-pig inclusion conjunctivitis (GPIC) eluted from SDS-polyacrylamide gels (SDS-MOMP, SDS-61 kDa). Post-immunization sera and secretions contained antibodies to the SDS-purified proteins at high titre as measured by immunoblotting, whereas enzyme immunoassays (EIA) using whole elementary bodies as antigen showed significantly lower titres ($P < 0{\cdot}001$). Likewise, blastogenic responses of peripheral mononuclear cells to GPIC elementary bodies were weak. Animals immunized with SDS-MOMP and SDS-61 kDa were fully susceptible to intravaginal challenge, as were control animals immunized with buffer without protein. Another group of animals were immunized with material prepared by extraction of chlamydial outer-membrane complexes with octyl β-D-glucopyranoside (OGP) and dithiothreitol, which consisted largely of MOMP (OGP-MOMP). In contrast to the SDS-MOMP group, sera and secretions in the OGP-MOMP group showed high titres in EIA, and high titre antibodies to MOMP by immunoblot; however, most animals also had antibodies to 61 kDa, 72 kDa and ca. 84 kDa outer-membrane proteins. OGP-MOMP animals were partially protected against genital challenge as evidenced by low inclusion scores compared to control animals, although duration of infection measured by culture isolation was similar to controls. Immunoblot analysis of sera from immunized animals and from a group of immune animals post-infection was performed using recombinant fusion peptides containing the four variable domains of MOMP. No consistent differences in reaction patterns were observed when sera from protected and non-protected animals were compared. Thus, a highly refined outer-membrane preparation is capable of producing partial immunity to genital infection. Further study is required to determine whether the protection is due to MOMP itself or to other outer-membrane proteins found in small amounts in the OGP-MOMP immunogen. The results suggest the possibility that discontinuous MOMP epitopes could play a role in inducing a protective immune response in the guinea-pig model, a concept that requires further evaluation.

Introduction

Development of a safe effective chlamydial vaccine has been considered an important goal because of the high prevalence and considerable morbidity associated with both trachoma and genital infections due to *Chlamydia trachomatis*. One difficulty in vaccine development is that strains of *C. trachomatis*, excepting the mouse pneumonitis (MoPn) strain, are strictly human pathogens. However, several animal models, including conjunctivitis models in primates (Taylor *et al.*, 1988), have proved useful in previous *C. trachomatis* immunization studies. The guinea-pig models of eye and genital infection caused by the naturally-occurring pathogen guinea-pig inclusion conjunctivitis agent (GPIC), a *C. psittaci* strain,

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Abbreviations: EB, elementary body; EIA, enzyme immunoassays; GPIC, guinea-pig inclusion conjunctivitis; MOMP, major outer-membrane protein; OGP, octyl β-D-glucopyranoside; VD, variable domain.

have been well-characterized (Batteiger & Rank, 1987; Rank & Batteiger, 1989; Rank *et al.*, 1988; Rank & Barron, 1987). The guinea-pig:GPIC models offer alternative and more accessible experimental systems in which to develop vaccine strategies. We know that a brief period of absolute protection against reinfection results from genital infection in this model, followed by a prolonged period of partial protection (Rank *et al.*, 1988). It is thus reasonable to determine if such protection can be induced artificially.

Another difficulty in vaccine development was noted in trachoma vaccine trials conducted in the 1960s (summarized in Schachter & Dawson, 1978) using inactivated whole elementary bodies. Some immunized patients experienced enhanced eye disease (hypersensitivity) as compared to unvaccinated controls. A GPIC genus-specific hypersensitivity protein capable of inducing such responses in the guinea-pig eye has been described (Morrison *et al.*, 1989). Thus, a subunit vaccine, which would separate protective from deleterious components, would be desirable (Schachter, 1985).

The chlamydial major outer-membrane protein (MOMP) has been most intensively studied as a potential immunogen. A cross-species analysis of available MOMP sequences has revealed a highly conserved primary structure with the exception of four variable domains (VD1–4) (Stephens *et al.*, 1988; Yuan *et al.*, 1989; Zhang *et al.*, 1989). Based on immunoblot analyses of sera from guinea-pigs with GPIC genital infection, VDs 1–4 appear to be immunodominant (B. E. Bavoil & R. G. Rank, unpublished data). These domains in *C. trachomatis* strains contain serovar-, subspecies- and species-specific determinants (Baehr *et al.*, 1988), some of which are neutralizing in *in vitro* systems (Baehr *et al.*, 1988; Zhang *et al.*, 1987). In addition, trypsin-sensitive sites in VD2 and VD4 have been implicated in chlamydial attachment (Su *et al.*, 1988). Collectively, these data support the use of MOMP in the development of a subunit vaccine. A vaccine consisting of chlamydial outer-membrane protein complexes protected sheep against systemic infection with the *Chlamydia psittaci* agent of ovine enzootic abortion (Tan *et al.*, 1990). However, MOMP purified in the presence of SDS did not induce substantial protective immunity when used as an oral immunogen in a monkey conjunctivitis model (Taylor *et al.*, 1988).

The aim of this study was to determine if isolated MOMP, prepared using either SDS or a nonionic detergent and then given parenterally, could induce a protective immune response in the guinea-pig:GPIC model of genital infection. Because antibodies to a cysteine-rich ca. 60 kDa outer-membrane protein are frequently induced in both human (Newhall *et al.*, 1982) and guinea-pig (Batteiger & Rank, 1987) genital infections, we chose to study the effect of this protein in the model as well.

Methods

Chlamydiae and protein purification. Elementary bodies (EBs) of the *Chlamydia psittaci* agent of guinea-pig inclusion conjunctivitis (GPIC) were produced by growth in McCoy cell monolayers and purified using Percoll gradients by established procedures (Batteiger & Rank, 1987). These EBs were used as starting material to purify proteins, and as antigen in immunoblot, EIA and blastogenesis assays. Chlamydiae for challenge infection were prepared in HeLa cells as previously described (Rank *et al.*, 1988). Chlamydial outer-membrane complexes of GPIC were produced using standard procedures (Batteiger *et al.*, 1985; Caldwell *et al.*, 1981) and used as starting material for production of purified proteins.

The first method used to obtain purified 39 kDa MOMP and the only method employed to obtain the 61 kDa cysteine-rich outer membrane protein was to first resolve outer-membrane proteins on 3 mm thick SDS-polyacrylamide gels (Caldwell & Schachter, 1982). The appropriate bands were then excised from the gel, diced into fragments, and eluted into Laemmli electrophoresis buffer (Laemmli, 1970) using an electroelution device (Schleicher and Schuell). Because these proteins were isolated in the presence of SDS, they were designated SDS-MOMP and SDS-61 kDa.

Purified MOMP was obtained without the use of SDS by the method of Bavoil *et al.* (1984) employing octyl β-D-glucopyranoside (OGP; Boehringer-Mannhein) and dithiothreitol (DTT). Chlamydial outer-membrane complexes were incubated in a solution containing 2% (w/v) Sarkosyl and 40 mM-DTT for 1 h at 37 °C followed by centrifugation at 100000 *g*. The supernatant contained most contaminating proteins including the 61 kDa, 11 kDa and ca. 84 kDa outer-membrane proteins of GPIC (Batteiger & Rank, 1987). The pellet was then incubated with a solution containing 2% (w/v) OGP and 40 mM-DTT for 1 h at 37 °C followed by centrifugation at 100000 *g*. The supernatant contained MOMP as the primary component. These MOMP preparations were designated OGP-MOMP.

The purity of all preparations was assessed using SDS-polyacrylamide gels stained with Coomassie blue R-250. Gels containing OGP-MOMP were scanned using a laser densitometer (LKB-Bromma), with the proportion of dye binding attributable to MOMP estimated by a programmable integrator (Hewlett-Packard). Protein contents were estimated by the dye-binding assay of Bradford (1976) (Bio-Rad). SDS-MOMP, SDS-61 kDa and OGP-MOMP were used to immunize female guinea-pigs as described below. Two lots of each antigen were prepared independently, and each lot was used to immunize five experimental animals.

Experimental animals, immunizations, and challenge infection. Hartley strain female guinea-pigs, weighing 450–500 g, were obtained from Sasco Laboratories and were housed individually in an environmentally controlled room with a 12 h light–dark cycle. Hartley strain guinea-pigs are outbred. For each experiment, groups of 10 animals were immunized with either 10 μg of a given purified protein preparation ($n = 5$) or mock-immunized with an equal volume of the relevant detergent-containing buffer ($n = 5$). Each animal received three subcutaneous immunizations, each 2 weeks apart. The primary immunization was with Freund's complete adjuvant whereas the second and third immunizations were with Freund's incomplete adjuvant. Each experiment was repeated once, so that a total of 10 animals each were immunized with SDS-MOMP, OGP-MOMP, or SDS-61 kDa, with corresponding mock-immunized control animals.

Guinea-pigs in all groups were challenged 2 weeks following the third immunization with viable HeLa cell-grown chlamydiae administered

Table 1. *Characteristics of plasmid constructs*

Plasmid	Insert	Product	Molecular mass (kDa)
pATH10	–	TrpE	37
pGM101	Asp141-Ala330	TrpE-MOMP	58
pAJC264	–	LamB	47·8
pGM1	Thr67-Arg80	LamB-VD1	49·3
pGM2	Leu135-Pro147	LamB-VD2	49·1
pGM3	Thr218-Ser232	LamB-VD3	49·2
pGM4-1	Pro285-Gly300	LamB-VD4-1	49·5
pGM4-2	Leu299-Ala312	LamB-VD4-2	49·2

intravaginally. Each animal received approximately 10^6–10^7 inclusion-forming units contained in 0·05 ml sucrose/phosphate/glutamate buffer, pH 7·4 (Rank *et al.*, 1988). The course of infection was followed by determining the percentage of inclusion-bearing cells on a Giemsa-stained smear of a scraping from the vaginal vault, and by determining the presence of viable GPIC organisms using cell culture (Rank *et al.*, 1988). Differences in the course of infection were determined using a two-factor (days, treatment group) analysis of variance with repeated measures of one factor (days).

Expression of recombinant MOMP epitopes. Regions of GPIC MOMP which correspond to the four variable domains (VDs) identified in MOMPs from *C. trachomatis* (Stephens *et al.*, 1988; Zhang *et al.*, 1987) were genetically inserted between serine residues 153 and 154 of the LamB protein of *E. coli* using the expression vector pAJC264 (Charbit *et al.*, 1986). Synthetic oligonucleotides encoding MOMP domains corresponding to VD1 (5′-GATCCGACCGGTAACGCTG-CAGCTGACTTTAAAACCGTTGCCGATCGT), VD2 (5′-GATCT-GGGTGTTACCGGTACCGACCTGCAGGGTCAGTACCCG), VD3 (5′-GATCCGACTGCAGCTAACTTCCCGCTGCCGCTGAC-CCCTACCCTCCTAGGT), VD4-1 (N-terminal end) (5′-GATCCTA-CCGCAATATTAAACCTGACCACCTGGAACCCTACCCTCC-TAGGT) and VD4-2 (C-terminal end) (5′-GATCTGGGTGAAGCT-ACCACCATTAATACCGGCGCCAAATACGCT) were designed to comply with the LamB gene preferred codon usage (Charbit *et al.*, 1986), and to generate two 5′-GATC overhangs to allow insertion into the *Bam*HI site of pAJC264. GPIC MOMP VDs were defined by comparison with the reported *C. trachomatis* VD sequences (Yuan *et al.*, 1989; Zhang *et al.*, 1989). More conservative amino acid substitutions were excluded from our design to allow synthesis of single pairs of oligonucleotides each for VD1 and VD2. In the case of VD4, two oligonucleotide pairs were made to generate two distinct hybrids, LamB-VD4-1 and LamB-VD4-2, with a two amino acid overlap (Table 1). The N-terminal VD4-1 and C-terminal VD4-2 correspond to the postulated subspecies-specific and serovar-specific domains of VD4 in *C. trachomatis* respectively (Stephens *et al.*, 1988). After religation of annealed pairs into the *Bam*HI site of pAJC264 and transformation of *E. coli* DH5α, recombinant plasmids were characterized by restriction and nucleotide sequence analyses. Expressed LamB-VD1, -VD2, -VD3 and -VD4-2 hybrid polypeptides were of the expected sizes with the expected molecular mass increments of 1·5 to 2·5 kDa and reacted strongly with polyclonal antisera against LamB and MOMP by immunoblot (Fig. 3). Hybrid polypeptide LamB-VD4-1 had a somewhat lower apparent molecular mass than expected (predicted 49·5 kDa). However, the presence of the appropriate VD4-1 insert in the LamB site was confirmed by nucleotide sequence analysis and by immunoblot.

A recombinant expressing a larger domain of GPIC MOMP was made by insertion of a *Bgl*II–*Hin*dIII fragment (nucleotides 486–1052 of the GPIC MOMP structural gene) into the *Bam*HI–*Hin*dIII restricted expression vector pATH10 (Spindler *et al.*, 1984). *E. coli* RR1 cells harbouring this plasmid construct (pGM101) express a 58 kDa hybrid polypeptide (TrpE-MOMP) which includes the 37 kDa N-terminal-most portion of the TrpE protein and a 21 kDa MOMP segment at the C-terminal end (Table 1, Fig. 3).

Assessment of response to immunization. Serum and genital secretions were obtained by established methods (Rank *et al.*, 1988) from each animal immediately prior to challenge infection, 2 weeks after the third immunization. Antibody titres in serum and genital secretions were determined by an enzyme immunoassay (EIA) using whole GPIC EBs (Rank *et al.*, 1988). Antigen-specific antibody binding was assessed initially using immunoblot analyses using whole EBs as antigen (Batteiger & Rank, 1987). GPIC EBs grown in McCoy cells were used as antigen in both EIA and immunoblot. Peripheral blood mononuclear cells were obtained prior to challenge infection, and blastogenic responses of such cells to whole GPIC EBs were determined according to established methods (Rank *et al.*, 1988).

The humoral responses against linear epitopes contained in the specific VDs of GPIC MOMP were assessed by immunoblot using cell lysates from each of the recombinants described above. Radioiodinated staphylococcal protein A was used to detect bound antibody. Antigen preparations were adjusted to contain approximately 1 μg of recombinant protein and sera were used at a single dilution (1:2000). Immunoblots were exposed for 24–48 h initially and then re-exposed for 10–14 d to confirm negative results.

Results

Purification of GPIC proteins

Fig. 1(*a*) shows a SDS-12·5% polyacrylamide gel containing resolved proteins of OGP-MOMP stained with Coomassie blue R-250. SDS-MOMP and SDS-61 kDa were single entities when similarly analysed (not shown). The OGP-MOMP (Fig. 1*a*) contained primarily MOMP, but other minor bands were visible, including bands in the region of the 84 kDa outer membrane proteins, and 47 kDa and 33 kDa outer membrane proteins (Batteiger & Rank, 1987). We estimated the purity of MOMP to be approximately 80% based on densitometric scanning of Coomassie blue-stained gels. Our results thus differ somewhat from those reported for *C. trachomatis* strain L_2/434 by Bavoil *et al.* (1984) who achieved > 90% purity assessed by a similar method. The more prominent contaminant bands observed here may have been due in part to differences in solubility of some outer membrane proteins in the *C. psittaci* strain as compared to the *C. trachomatis* strain.

Immune response of immunized guinea-pigs

Fig. 1(*b*, *c*) depicts immunoblots showing the qualitative responses of five animals to immunization with each of the two MOMP preparations from Experiment 2 (Table 2). Mock-immunized animals were negative by immunoblot (not shown).

Fig. 1(*b*) shows the serum IgG response by immunoblot of the five animals immunized with the SDS-MOMP

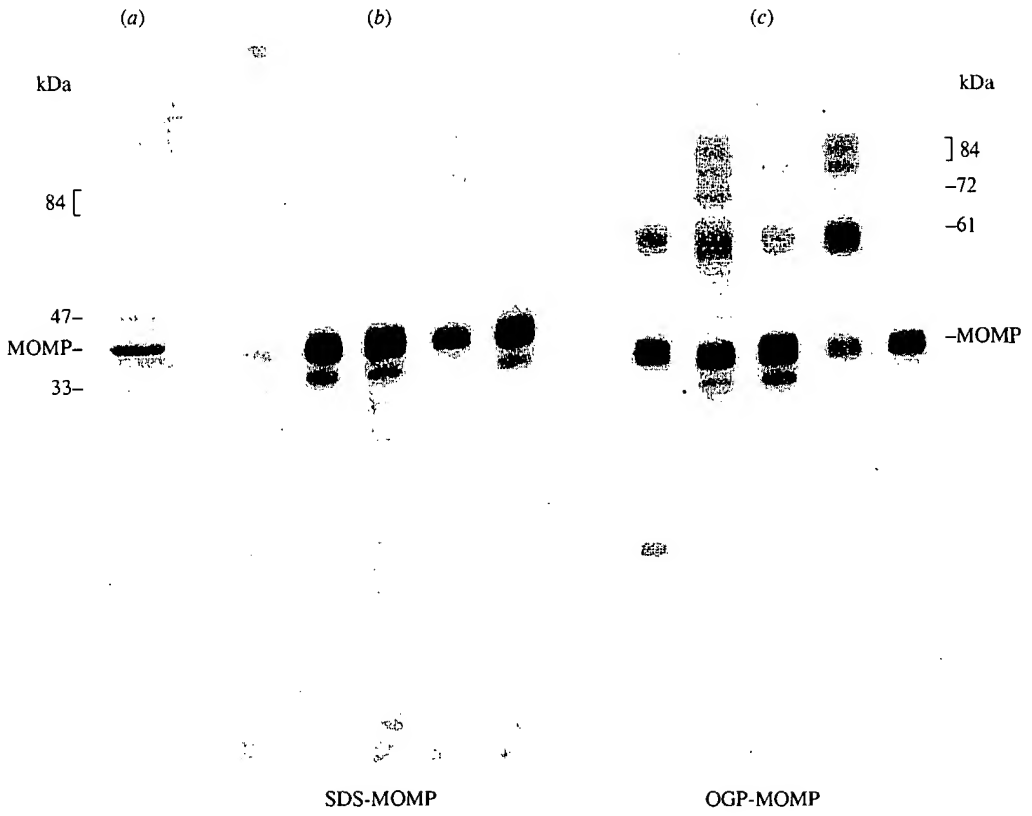

Fig. 1. (*a*) Coomassie blue-stained 12·5% polyacrylamide gel containing resolved proteins of chlamydial major outer membrane protein (MOMP) isolated using octyl-β-D-glucopyranoside (OGP) as described in the text. Identity of bands are noted on the left. (*b*) Autoradiogram of the serum IgG responses of the five guinea-pigs from Experiment 2 which were immunized with MOMP isolated from SDS-PAGE gels. (*c*) Autoradiogram of the serum IgG responses of the five animals from Experiment 2 which were immunized with OGP-MOMP. Identity of bands are marked on the right.

Table 2. *Immune responses in immunized animals*

Antibody titres are expressed as mean $\log_{10}$ (standard deviation).

Group	Experiment	Serum IgG EIA	Serum IgG immunoblot	Secretion IgG EIA	Secretion IgA EIA	Lymphocyte proliferation†
OGP-MOMP	1	3·2* (0·25)	4·01	2·1* (0·25)	ND	24290 (20381)
	2	4·01*	4·01	3·6* (0·27)	3·0* (0·16)	ND
SDS-MOMP	1	0·40 (0·55)	3·5 (0·58)	0	ND	8738 (2046)
	2	1·8 (0·25)	3·9 (0·13)	1·2 (0·13)	0·8 (0·5)	ND
SDS-61	3	0·40 (0·55)	⩾ 2·0	ND	ND	ND
	4	1·2 (0·16)	⩾ 2·0	ND	ND	12946 (10397)
Controls	1–4	0	0	0	0 (1374)	2440

ND, Not Determined.
* $P < 0·001$ compared to SDS-MOMP group.
† Counts per minute (standard deviation).

preparation in Experiment 2. All animals responded by producing antibodies that bound MOMP; however, one animal responded only weakly. IgG in secretions (not shown) paralleled that of sera. A second band was sometimes seen at a position in the gel corresponding to about 35 kDa (Fig. 1*b*). We do not know whether this

Table 3. *Reactivity of sera from immunized and infected guinea-pigs against recombinant MOMP fragments*

Number of positive sera/number of animals tested in each group. Sera were called negative only when the level of reactivity remained undetectable above background after a prolonged exposure (see text).

	Guinea-pigs immunized with SDS-MOMP	Guinea-pigs immunized with OGP-MOMP	Guinea-pigs infected in the genital tract
TrpE	0/5	0/4	0/5
TrpE-MOMP	5/5	4/4	25/29
LamB	0/9	1/4	0/29
LamB-VD1	8/9	4/4	26/29
LamB-VD2	3/9	3/4	24/29
LamB-VD3	6/9	3/4	2/4
LamB-VD4-1	6/9	4/4	3/4
LamB-VD4-2	6/9	3/4	6/29

band represented antibodies formed in response to a protein contaminating the immunogen, or MOMP antibodies that bound an altered (e.g. proteolytically degraded or internally disulphide-linked) form of MOMP.

Fig. 1 (*c*) shows the serum IgG responses by immunoblot of five animals immunized with OGP-MOMP in Experiment 2. All sera contained antibodies to MOMP and also to the 35 kDa band described above. In addition, 4/5 (9/10 overall) gave responses to the 61 kDa outer membrane protein, and 3/5 (8/10 overall) to the ca. 84 kDa outer membrane proteins. These results indicate that the OGP-MOMP preparation was contaminated with enough 61 kDa to elicit a detectable IgG response, but not to give a visible band in Coomassie-stained gels. Although the 47 kDa and 33 kDa proteins were visible contaminants by SDS-PAGE analysis, they did not elicit an antibody response detectable by immunoblot. In Experiment 1, 5/5 animals in the OGP-MOMP group had binding at the region of LPS, suggesting that LPS was present in the first lot of immunogen. However, in Experiment 2 (Fig. 1 *c*), no sera bound antibody at the region of LPS.

Quantitative humoral immune responses for all animals both by EIA and immunoblot are given in Table 2. When assessed by immunoblot, there was no statistical difference in the uniformly high antibody titres against MOMP between animals immunized with OGP-MOMP and SDS-MOMP (Table 2). In contrast, when assessed by whole EB EIA, the OGP-MOMP preparation elicited a significantly higher antibody response than the SDS-MOMP preparation, both in serum and secretions ($P < 0{\cdot}001$, Table 2). Antibody titres measured by EIA and immunoblot were not statistically different in the OGP-MOMP group, whereas in the SDS-MOMP group,

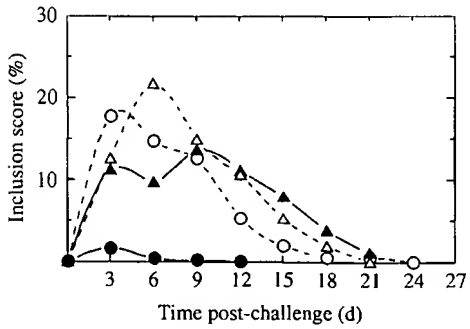

Fig. 2. Course of genital infection of groups (10 animals in each group) immunized with OGP-MOMP (●), OGP-control (○), SDS-MOMP (▲), and SDS-control (△). OGP- and SDS-controls were mock immunized with detergent in buffer only. Inclusion scores were determined as described in the text and are expressed as a percentage of epithelial cells examined. Lack of a symbol at any given day indicates that inclusions were not found in the genital smears from that day.

EIA titres were significantly lower than immunoblot titres ($P < 0{\cdot}001$).

While the blastogenic responses were higher in the OGP-MOMP group than in the SDS-MOMP group, the difference in the mean response was not statistically significant (Table 2).

Animals immunized with SDS-61 kDa had serum IgG responses against the 61 kDa protein at titres $\geqslant 1:100$ in immunoblot. Titres in EIA are shown in Table 2.

Response to challenge infection

The summed courses of infections for the 10 animals receiving each treatment is shown in Fig. 2. The course of infection in groups of animals immunized with either SDS-MOMP or SDS-61 kDa did not differ from the infections observed in the control groups. Thus, no protection was observed despite the immune responses described above. In contrast, the group of 10 animals immunized with OGP-MOMP had significantly reduced intensity and duration of infection as measured by inclusion scores ($P < 0{\cdot}0001$), although duration of chlamydial shedding detected by cell culture did not differ from the control group. We saw no difference in the courses of infection in the OGP-MOMP groups in Experiment 1 (with LPS antibodies) versus Experiment 2 (no LPS antibodies).

Fine specificity of serum antibodies to MOMP

Recombinant *E. coli* cells expressing LamB-VD1, -VD2, -VD3 and LamB fused to VD4-1 (N-terminal half) and VD4-2 (C-terminal half) served as antigen in immunoblots to analyse the specificity of serum antibodies from guinea pigs immunized with either OGP-MOMP or SDS-MOMP. In addition, sera from 29 animals which

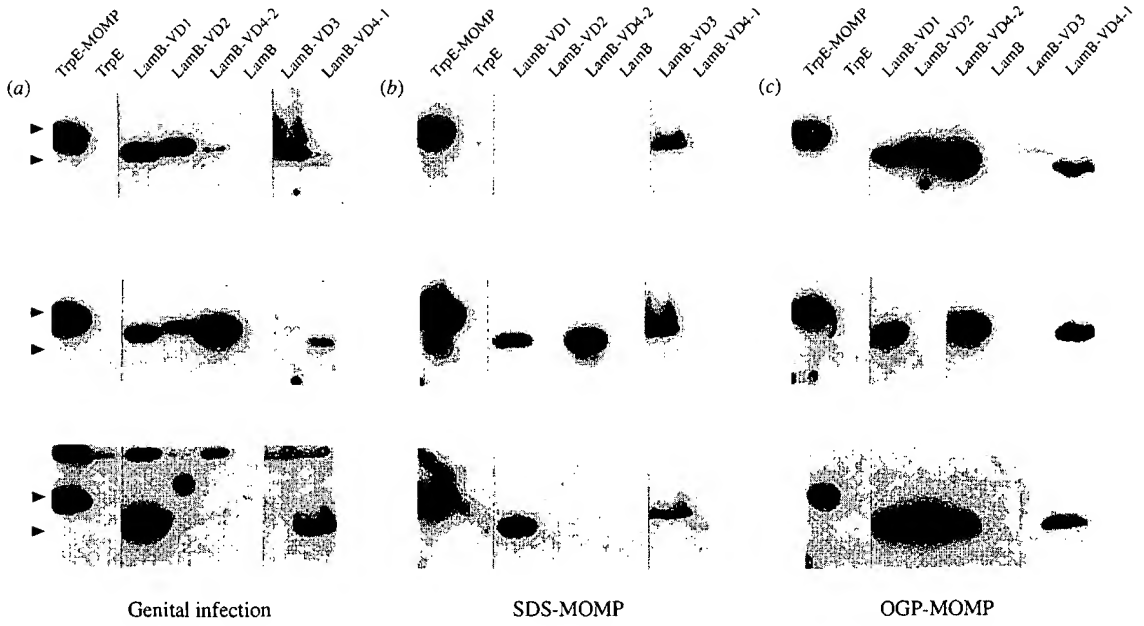

Fig. 3. Autoradiograms of IgG reactions in immunoblots of representative sera to recombinant peptides representing the variable domains of the MOMP of the guinea-pig inclusion conjunctivitis agent. Panels *a*, *b* and *c* represent the results of a serum from each of three animals used to probe a panel of eight recombinant antigens from *E. coli* lysates. (*a*) Sera from three animals which recovered from a genital infection and were thus partially protected against challenge infection. (*b*) Sera from three animals immunized with SDS-MOMP and were thus unprotected against challenge infection. (*c*) Sera from three animals immunized with OGP-MOMP and were thus partially protected against challenge infection. Arrowheads on the left indicate the positions of the fusion proteins containing chlamydial antigens.

had recovered from a genital infection and were resistant to re-challenge (Rank *et al.*, 1988) were included for comparison.

Sera from all immunized animals gave strong reactions with TrpE-MOMP (Table 3), confirming results obtained with whole MOMP (Fig. 1), while 25/29 infected animals gave reactions to TrpE-MOMP (Table 3). Reactions of sera with the various recombinant VDs were not uniform (Table 3) and included sera from each group which did not react with one or more of the recombinant peptides (Fig. 3). Table 3 shows that animals immunized with SDS-MOMP reacted less frequently to LamB-VD2 as compared to animals in the OGP-MOMP-immunized group, while the group of animals which had recovered from genital infection reacted less frequently to LamB-VD4-2. However, the analysis failed to reveal statistically significant differences in reactivity among the three groups of sera for any recombinant peptide.

Discussion

Our results indicate that a protective immune response can be elicited in the guinea-pig:GPIC model of genital chlamydial infection using a refined vaccine (OGP-MOMP) derived from outer membrane complexes. The vaccine was largely MOMP, but in most animals elicited antibodies observed by immunoblot that bound other outer membrane components, including the 61 kDa, 84 kDa and 72 kDa outer membrane proteins. Genital infection with the GPIC agent frequently induces antibodies to these outer membrane proteins (Batteiger & Rank, 1987). Thus, we cannot conclusively determine whether the observed protection was the result of immune responses elicited by MOMP itself, or the result of responses elicited by contaminant outer membrane proteins. However, it should be noted that one animal in the OGP-MOMP group had a monospecific response to MOMP and showed the same degree of protection as the other animals. In either case, we have shown that a parenterally administered subunit vaccine can induce detectable protection against mucosal infection as defined by a marked decrease in intensity of infection. Histopathology of the lower and upper genital tract was not evaluated as a part of this study.

Our results also indicate that SDS-MOMP, as prepared and administered in this study, was capable of inducing antibodies to MOMP in both serum and secretions, but incapable of inducing protection. Likewise, a vaccine prepared from SDS-purified 61 kDa outer membrane protein induced measurable immune responses, but afforded no protection.

The SDS-MOMP vaccine elicited strong antibody

responses that could be measured by immunoblot, but only weak antibody responses measured by EIA. Blastogenic responses were also weak. The EIA assay, using whole EBs as antigen, detects primarily antibodies to surface-exposed antigens. It is possible that the SDS-MOMP vaccine elicited responses to cross-reactive but surface inaccessible antigens which are not involved in eliciting a protective response. The same patterns held for the SDS-61 kDa immunogen. The analogue of the latter protein has been shown not to be surface exposed in *C. trachomatis* strains (Collett *et al.*, 1989), even though it frequently induces a prominent humoral response in genital infection in humans (Newhall *et al.*, 1982) and guinea-pigs (Batteiger & Rank, 1987).

In contrast, the OGP-MOMP elicited strong antibody responses both in immunoblot and in EIA, and elicited marginally more vigorous blastogenic responses. As compared to SDS-MOMP, it is possible that MOMP in the OGP preparation has a conformation more closely approximating that of its native configuration and thus either elicited a protective response to discontinuous epitopes or a more efficient response to protective linear epitopes.

In an attempt to identify regions of MOMP differentially recognized by anti-MOMP antibodies from unprotected (SDS-MOMP-immunized) and protected (post-infection and OGP-MOMP-immunized) animals, recombinants expressing peptides corresponding to the immunodominant VDs of MOMP were used to analyse the sera. While virtually all antisera from all three groups contained high titres of antibodies to whole and recombinant MOMP by immunoblot, responses to individual VDs were more variable. Thus, there were no VD regions to which all protected animals responded but to which no unprotected animals responded. Although the proportions of animals with reactive sera varied by group, no statistically significant differences and thus no striking correlations of reactivity and protection were found.

The observed protection was only partial, in that organisms were shed, albeit at low levels, even in the OGP-MOMP animals. The reason for this is not clear but could be related to an inability of the subcutaneous vaccine to elicit an adequate cell-mediated response. This could result either from a failure to elicit appropriate effector cells or failure to elicit those T cells which are able to home to the mucosa-associated lymphoid tissue in the genital tract. We have previously demonstrated that cell-mediated immunity is essential for the resolution of a chlamydial genital infection (Rank *et al.*, 1989). Absolute prevention of infection may be difficult to achieve, since even in the natural infection, such immunity is short-lived (Rank *et al.*, 1988). A more appropriate goal might be the reduction of the level of infection and prevention of ascending infection resulting in salpingitis. Thus, studies are in progress to determine whether subunit immunizations influence the occurrence of such complications in female guinea-pigs.

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References

BAEHR, W., ZHANG, Y.-X., JOSEPH, T., SU, H., NANO, F. E., EVERETT, K. D. E. & CALDWELL, H. D. (1988). Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 4000–4004.

BATTEIGER, B. E. & RANK, R. G. (1987). Analysis of the humoral immune response in chlamydial genital infection in guinea pigs. *Infection and Immunity* **55**, 1767–1773.

BATTEIGER, B. E., NEWHALL, W. J. V. & JONES, R. B. (1985). Differences in outer membrane proteins of the lymphogranuloma venereum and trachoma biovars of *Chlamydia trachomatis*. *Infection and Immunity* **50**, 488–494.

BAVOIL, P., OHLIN, A. & SCHACHTER, J. (1984). Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. *Infection and Immunity* **44**, 479–485.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

CALDWELL, H. D. & SCHACHTER, J. S. (1982). Antigenic analysis of the major outer membrane protein of *Chlamydia* spp. *Infection and Immunity* **35**, 1024–1031.

CALDWELL, H. D., KROMHOUT, J. & SCHACHTER, J. S. (1981). Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infection and Immunity* **31**, 1161–1176.

CHARBIT, A., BOULAIN, J.-C., RYTER, A. & HOFNUNG, M. (1986). Probing the topology of a bacterial membrane protein by genetic insertion of a foreign epitope: expression at the cell surface. *EMBO Journal* **5**, 3029–3037.

COLLETT, B. A., NEWHALL, W. J. V., JERSILD, R. A., JR & JONES, R. B. (1989). Detection of surface-exposed epitopes on *Chlamydia trachomatis* by immune electron microscopy. *Journal of General Microbiology* **135**, 85–94.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

MORRISON, R. P., LYNG, K. & CALDWELL, H. D. (1989). Chlamydial disease pathogenesis: ocular hypersensitivity elicited by a genus-specific 57-kD protein. *Journal of Experimental Medicine* **169**, 663–675.

NEWHALL, W. J. V., BATTEIGER, B. E. & JONES, R. B. (1982). Analysis of the human serological response to proteins of *Chlamydia trachomatis*. *Infection and Immunity* **38**, 1181–1189.

RANK, R. G. & BARRON, A. L. (1987). Specific effect of estradiol on the genital mucosal antibody response in chlamydial ocular and genital infections. *Infection and Immunity* **55**, 2317–2319.

RANK, R. G. & BATTEIGER, B. E. (1989). Protective role of serum antibody in immunity to chlamydial genital infection. *Infection and Immunity* **57**, 299–301.

RANK, R. G., BATTEIGER, B. E. & SODERBERG, L. S. F. (1988). Susceptibility to reinfection after a primary chlamydial genital infection. *Infection and Immunity* **56**, 2243–2249.

RANK, R. G., SODERBERG, L. S. F., SANDERS, M. M. & BATTEIGER, B. E. (1989). Role of cell-mediated immunity in the resolution of secondary chlamydial genital infection in guinea pigs infected with

the agent of guinea pig inclusion conjunctivitis. *Infection and Immunity* **57**, 706–710.

SCHACHTER, J. (1985). Overview of *Chlamydia trachomatis* infection and the requirements for a vaccine. *Reviews of Infectious Diseases* **7**, 713–716.

SCHACHTER, J. & DAWSON, C. R. (1978). *Human Chlamydial Infections*. Littleton, Mass: PSG Publishing Co.

SPINDLER, K. R., ROSSER, D. S. E. & BERK, A. J. (1984). Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. *Journal of Virology* **49**: 132–141.

STEPHENS, R. S., WAGAR, E. A. & SCHOOLNIK, G. K. (1988). High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of *Chlamydia trachomatis*. *Journal of Experimental Medicine* **167**, 817–831.

SU, H., ZHANG, Y.-X., BARRERA, O., WATKINS, N. & CALDWELL, H. D. (1988). Differential effect of trypsin on infectivity of *Chlamydia trachomatis*: loss of infectivity requires cleavage of major outer membrane protein variable domains II and IV. *Infection and Immunity* **56**, 2094–2100.

TAN, T.-W., HERRING, A. J., ANDERSON, I. E. & JONES, G. E. (1990). Protection of sheep against *Chlamydia psittaci* infection with a subcellular vaccine containing the major outer membrane protein. *Infection and Immunity* **58**, 3101–3108.

TAYLOR, H. R., WHITTUM-HUDSON, J., SCHACHTER, J., CALDWELL, H. D. & PRENDERGAST R. A. (1988). Oral immunization with chlamydial major outer membrane protein (MOMP). *Investigative Ophthalmology and Visual Science* **29**, 1847–1853.

YUAN, Y., ZHANG, Y.-X., WATKINS, N. G. & CALDWELL, H. D. (1989). Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. *Infection and Immunity* **57**, 1040–1049.

ZHANG, Y.-X., STEWART, S., JOSEPH, T., TAYLOR, H. R. & CALDWELL, H. D. (1987). Protective monoclonal antibodies recognize epitopes located on the major outer membrane protein of *Chlamydia trachomatis*. *Journal of Immunology* **138**, 575–581.

ZHANG, Y.-X., MORRISON, S. G., CALDWELL, H. D. & BAEHR, W. (1989). Cloning and sequence analysis of the major outer membrane protein genes of two *Chlamydia psittaci* strains. *Infection and Immunity* **57**, 1621–1625.

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Immunization with an Acellular Vaccine Consisting of the Outer Membrane Complex of *Chlamydia trachomatis* Induces Protection against a Genital Challenge

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The ability to induce protection against a genital challenge was studied in BALB/c female mice with three *Chlamydia trachomatis* mouse pneumonitis (MoPn) major outer membrane protein (MOMP) preparations as well as an acellular vaccine consisting of the chlamydial outer membrane complex (COMC). The MOMP preparations were extracted with three different types of detergents, sodium dodecyl sulfate (SDS), *n*-octyl-β-D-glucopyranoside (OGP), and Zwittergent 3-14 (Z3-14). A positive immunization control consisted of mice inoculated intranasally with 10^4 *C. trachomatis* MoPn inclusion-forming units (IFU). Mice inoculated with ovalbumin served as a negative control. Furthermore, a sham-immunized, nonchallenged group was included as a fertility control. Two weeks after the last immunization, the mice were challenged in the left ovarian bursa with 10^5 *C. trachomatis* MoPn IFU. Vaginal swabs were collected for culture, vaginal and serum samples were assayed for chlamydial-specific antibodies, and splenocytes were collected to determine the lymphoproliferative response. At 42 days after the challenge, the mice were mated with proven male breeder mice. Animals that were considered to be pregnant (as determined by weight) were killed, and the embryos were counted. A significant humoral and cell-mediated immune response was observed in all the groups of mice inoculated with chlamydial antigens. Antibodies to variable domain (VD)1 of the MOMP were detected in serum samples from all the immunized groups. However, antibodies to VD3 and VD4 were detected only in the groups immunized with the Z3-14–MOMP and the COMC. Mice immunized with COMC developed significant immunoglobulin A chlamydia-specific antibodies in the vagina, while mice immunized with the detergent-extracted MOMPs had low antibody titers. Following the intrabursal challenge, a significant decrease in the intensity and duration of vaginal shedding was noted in the mice immunized with COMC and a moderate decrease was noted in the group immunized with OGP-MOMP. No protection against the infection was noted in the groups of animals immunized with SDS- and Z3-14–MOMP. Furthermore, of the mice immunized with the COMC preparation, only 25% (4 of 20) shed *C. trachomatis*, as determined by vaginal culture, while 83% (40 of 48) of the control mice inoculated with ovalbumin were culture positive ($P < 0.05$). In addition, after mating, the mice inoculated with COMC were found to have fertility rates comparable to those of the control sham-immunized, nonchallenged animals (70% [14 of 20] versus 81% [17 of 21], respectively [$P > 0.05$]), and there were no significant differences between the average number of embryos per mouse in the two groups (5.1 versus 5.9, respectively [$P > 0.05$]). In contrast, mice immunized with the purified MOMP preparations were not protected against infertility. In summary, a preparation of the COMC protected mice against infection and infertility, supporting the feasibility of the development of an acellular vaccine against *C. trachomatis* infections.

Pelvic inflammatory disease (PID) is a major health problem throughout the world (10, 16, 31, 43–45). For example, in the United States, it is estimated that approximately 1 million women experience an episode of PID and that of these women, 200,000 are hospitalized yearly (10). In addition to the health problem, this creates an enormous economic burden, so it is not surprising that for the year 2000, the costs related to the care of individuals with PID has been projected to reach $10 billion in the United States (42).

Among the different microorganisms involved in PID, *Chlamydia trachomatis* plays a key role in a large number of cases (10, 31, 44, 45). The prevalence of chlamydial infections in Western countries has been estimated to range from 3 to 5% in asymptomatic women to 20 to 30% in women attending clinics for sexually transmitted diseases. In most areas of the world, *C. trachomatis* is the most common sexually transmitted bacterial pathogen and affects the female population mainly during its reproductive years (4, 6, 7, 31, 43–45). Approximately 4 million chlamydial infections occur in the United States on a yearly basis; 2.6 million affect women, 1.8 million affect men, and the rest involve infants (43). *C. trachomatis* can produce a wide range of clinical infections, including cervicitis and salpingitis in women, urethritis and epididymitis in men, and conjunctivitis and pneumonia in children (31). Although in most instances *C. trachomatis* produces a self-limited infection, in certain individuals the disease leads to chronic sequelae. Among the major complications that have been frequently associated with a *C. trachomatis* infection are tubal infertility, ectopic pregnancy, and chronic abdominal pain (4, 6, 16, 27, 31, 44, 45). Furthermore, over 50% of the infections in females are asymptomatic, and thus only preventive therapy can be effective against this disease (2, 12, 14, 16, 17, 41). As a result, there is a great need to develop a vaccine to prevent the transmission of *C. trachomatis*.

The major outer membrane protein (MOMP) of *C. trachomatis* has been considered by several authors to be the most likely candidate for an acellular vaccine (2, 14, 34, 41). Studies

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with humans and animal models indicate that the MOMP is strongly antigenic and elicits neutralizing antibodies. In humans, for example, Brunham et al. (7) analyzed the serological response in 52 patients with postabortion *C. trachomatis* salpingitis and showed that all the patients developed serum immunoglobulin A (IgA) antibodies and that 71% of them developed IgG antibodies to MOMP. Work by Caldwell et al. (8) and Caldwell and Schachter (9) with purified MOMP demonstrated that this protein can elicit an immune response in both mice and rabbits with species-, subspecies-, and type-specific antigenic determinants. Here, we tested four acellular vaccines containing the MOMP for their abilities to protect against infection and disease, e.g., infertility.

MATERIALS AND METHODS

Organism. The *C. trachomatis* mouse pneumonitis (MoPn) biovar (strain Nigg II) was purchased from the American Type Culture Collection (Rockville, Md.) and grown in HeLa-229 cells. Elementary bodies (EB) were purified as described by Caldwell et al. (8). Stocks of the organisms were frozen at −70°C in a solution containing 0.2 M sucrose, 20 mM sodium phosphate (pH 7.2), and 5 mM glutamic acid (SPG) (23, 25). The stocks were titered with HeLa-229 cells by centrifugation (21).

Preparation of vaccine candidates. The four vaccine candidates were prepared as follows.

(i) SDS-MOMP. Approximately 1.2 to 1.5 mg of purified *C. trachomatis* MoPn EB was resuspended in a solution containing 1 ml of 4% sodium dodecyl sulfate (SDS; Sigma Chemical Co., St. Louis, Mo.), 12% (wt/vol) glycerol, 50 mM Tris HCl (pH 6.8), 2% (vol/vol) β-mercaptoethanol, and 0.005% bromophenol blue, boiled for 10 min, and electrophoresed in a preparative polyacrylamide-tricine gel (8, 32). The proteins were visualized by submerging the gel for 5 min in 4 M sodium acetate. The band corresponding to 40 kDa was cut and electroeluted in a dialysis membrane with 0.02 M glycine and 0.0025 M Tris HCl, pH 8.3. The sample was dialyzed against phosphate-buffered saline (PBS; 0.01 M, pH 7.4) and stored at −80°C until used for vaccination.

(ii) OGP-MOMP. *C. trachomatis* EB were processed by following the procedures published by Bavoil et al. (3). Briefly, EB were resuspended by sonication in PBS with 1% Sarkosyl (International Biotechnologies, Inc., New Haven, Conn.) and incubated for 30 min at 37°C with mixing every 5 min. Insoluble material was pelleted at 100,000 × *g* for 1 h, and a second extraction of the pellet was performed as described above with the addition of 0.01 M dithiothreitol (DTT). The sample was centrifuged again, and the insoluble fraction was extracted with 1% *n*-octyl-β-D-glucopyranoside (OGP; Calbiochem-Novabiochem Corp., La Jolla, Calif.) and 0.01 M DTT. Following centrifugation, the supernatant was collected and dialyzed against 0.1% OGP in PBS, pH 7.4, before immunization.

(iii) Z3-14–MOMP. The MOMP was extracted with the zwitterionic detergent Zwittergent 3-14 (Z3-14; Calbiochem-Novabiochem Corp.) (8). EB were resuspended in 0.2 M phosphate buffer (pH 5.5) containing 0.001 M of EDTA and of phenylmethylsulfonyl fluoride (Sigma Chemical Co.). The detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Calbiochem-Novabiochem Corp.) at a final concentration of 2% and 0.1 M DTT were added. The sample was extracted for 2 h at 37°C, with constant mixing, and pelleted for 1 h at 100,000 × *g*. A second extraction was performed for 1 h under the same conditions, and the resulting pellet was extracted for 2 h at 37°C with 2% Z3-14, with constant mixing. The supernatant was harvested, and the MOMP was purified in a hydroxylapatite column at room temperature (8). The column was equilibrated with 0.02 M phosphate buffer (pH 5.5) containing 0.1% Z3-14 and 0.001 M each of EDTA, phenylmethylsulfonyl fluoride, and DTT. The column was eluted with a linear gradient of phosphate buffer from 0.02 to 0.5 M containing the same compounds as the equilibration buffer. The fractions containing the MOMP were pooled and dialyzed against PBS and 0.05% Z3-14.

(iv) COMC. The chlamydial outer membrane complex (COMC) was prepared by following the procedure described by Tan et al. (38). EB were incubated in a solution containing 0.1 M phosphate buffer (pH 7.4), 0.001 M EDTA, and 2% Sarkosyl for 1 h at 37°C, with mixing every 5 min. The insoluble material was pelleted by centrifugation at 100,000 × *g* for 1 h, the pellet was resuspended in the above-mentioned buffer, DTT was added to a concentration of 0.01 M, and the sample was incubated for 1 h at 37°C, with mixing every 5 min. The COMC was pelleted by centrifugation as described above and resuspended in PBS (pH 7.4). Glutaraldehyde was added to a final concentration of 0.1%, and the reaction was stopped after 2 min of incubation at room temperature by the addition of glycine to a concentration of 0.1 M (38). The cross-linked COMC was centrifuged for 1 h at 100,000 × *g* and resuspended in PBS containing 0.05% Z3-14.

A gel (10% tricine–SDS-polyacrylamide gel electrophoresis) was run with the different immunogens and stained with a silver stain kit (LabLogix, Belmont, Calif.) (22, 32).

Immunization and challenge protocols. Seven- to eight-week-old female BALB/c ($H\text{-}2^d$) mice were purchased from Simonsen Laboratory (Gilroy, Calif.). The animals were housed in isolation cubicles at a constant temperature of 24°C with a cycle of 12 h of light and 12 h of darkness and were fed mouse chowder ad libitum. All animal protocols were approved by the University of California, Irvine, Animal Care and Use Committee.

Mice were immunized with the different *C. trachomatis* MoPn MOMP preparations as follows. Mice were inoculated twice subcutaneously with 10 μg of SDS-MOMP, OGP-MOMP, or Z3-14–MOMP the first time with complete Freund's adjuvant and the second time 2 weeks later with incomplete Freund's adjuvant. The COMC preparation was adsorbed with 0.3% aluminium hydroxide solution (Serva GmbH & Co., Heidelberg, Germany) and mixed at a 1:1 ratio with incomplete Freund's adjuvant (38). Mice were boosted twice with the same dose at 2-week intervals. All the groups of mice were challenged on the 6th week after the first immunization. The positive control mice were inoculated intranasally with 10^4 *C. trachomatis* MoPn inclusion-forming units (IFU), and the negative controls received 10 μg of ovalbumin by the same protocol as the one used for the animals immunized with the MOMP preparations. Each time a MOMP preparation was tested, a positive control—mice immunized intranasally with *C. trachomatis* MoPn EB—and a negative control—mice inoculated with 10 μg of ovalbumin—were included in the study. A sham-immunized, nonchallenged group was included as a fertility control. All the experiments were repeated two or three times over a 3-year period. For data analysis, all the results obtained with the positive and negative control groups were added and used for statistical analyses.

For intrabursal inoculation, the animals were anesthetized with methoxyflurane and a lateral abdominal incision was made. The experimental group received 10^5 *C. trachomatis* MoPn IFU in 20 μl of SPG in the left ovarian bursa and mock-infected HeLa-229 cell extracts processed by the same protocol used to purify the EB in the right ovarian bursa (22, 37).

Six weeks after the intrabursal challenge, groups of four female mice were caged with a proven breeder male mouse for 18 days. Pregnancy was assessed by determining the increase in weight of the female mice starting at 10 to 12 days postmating as previously described (22). Animals that did not become pregnant were mated a second time (22).

Vaginal cultures. For the isolation of *C. trachomatis* MoPn, vaginal swabs were collected at regular intervals following the challenge. The specimens were cultured in McCoy cells grown in 24-well plates that were centrifuged at 1,000 × *g* for 1 h at room temperature (21, 22). At 48 h after inoculation, the monolayers were washed with PBS and fixed with methanol. The chlamydial inclusions were stained as previously described with a rabbit polyclonal anti-*C. trachomatis* MoPn serum prepared in our laboratory (21, 22).

Immunoassays. Blood was collected by periorbital or heart puncture, and the serum was separated by centrifugation and pooled for each group of animals. Vaginal washes were collected by irrigation of the vagina twice with 20 μl of PBS and were pooled for each group (11). All immunoassays were performed with the pooled serum and vaginal washes from each group. The *C. trachomatis* MoPn-specific antibody titer in serum and vaginal washes was determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (21, 22). Briefly, 96-well plates were coated with *C. trachomatis* MoPn EB in PBS at a concentration of 10 μg/ml, and 100 μl of serum or 50 μl of vaginal wash was added per well in twofold serial dilutions. After incubation at 37°C for 1 h and washing, the enzyme immunoassay plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, IgM, or IgA (Southern Biotechnology Associates, Inc., Birmingham, Ala.). The binding was measured in an ELISA reader (Bio-Rad Laboratories, Richmond, Calif.). For color development, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was used as the substrate.

The ability of the serum to neutralize the infectivity of *C. trachomatis* MoPn EB in vitro was determined as follows (26). *C. trachomatis* MoPn (10^4 IFU) was added to serial dilutions of the serum made with 5% guinea pig sera in PBS. After incubation at 37°C for 45 min, the mixture was inoculated by centrifugation onto HeLa-229 cells (22, 26). The monolayers were incubated for 24 h at 37°C in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, gentamicin (50 μg/ml), and cycloheximide (1 μg/ml). The preparations were fixed with methanol and stained with a rabbit polyclonal anti-*C. trachomatis* MoPn serum and a goat anti-rabbit peroxidase stain. IFU were counted at a magnification of ×200, and the 50% inhibition was calculated based on the sera from the animals inoculated with ovalbumin.

For Western blotting, *C. trachomatis* MoPn EB and the affinity-purified 60-kDa heat shock protein (hsp), kindly supplied by R. Morrison, (Rocky Mountain Laboratory, Hamilton, Mont.), were resolved by 10% tricine–SDS-polyacrylamide gel electrophoresis (32). Approximately 250 μg of purified EB was loaded on a 7.5-cm-wide slab gel. Following transfer to nitrocellulose membranes, the nonspecific sites were blocked with BLOTTO (Bovine Lacto Transfer Technique Optimizer: 5% [wt/vol] nonfat dried milk, 2 mM $CaCl_2$, and 50 mM Tris HCl [pH 8.0]), and the serum samples, diluted 1:200, were incubated at room temperature. The antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse antibody developed with 0.01% hydrogen peroxide and 4-chloro-1-napthol (21, 22).

A kit (Cambridge Research Biochemical, Cambridge, England) based on the technique developed by Geysen et al. (15) was used to map the binding sites of the mouse antibodies on overlapping octameric peptides representing the *C. trachomatis* MoPn MOMP amino acid sequence (13). Goat anti-mouse IgG

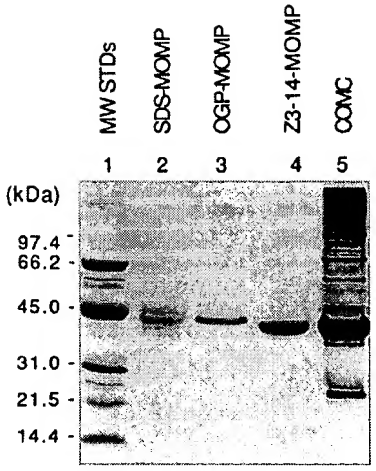

FIG. 1. Silver-stained gel of vaccine candidates. MW STDs, molecular weight standards.

conjugated to horseradish peroxidase served as the secondary antibody. The reactions were measured 1 h after ABTS was added by an ELISA reader equipped with a 405-nm filter.

Lymphocyte proliferation assay. To perform this assay, the spleens of two to four mice from each group were collected, teased, and enriched for T cells by passage over a nylon wool column. Accessory cells for antigen presentation were prepared by irradiating (3,000 rads; ^{137}Cs) syngenic unseparated spleen cells and incubating them at various ratios with *C. trachomatis* MoPn EB, and the lymphocyte proliferation assay was performed as previously described (21, 22). Concanavalin A (ConA) was used as a positive stimulant. At the end of 4 days of incubation, 1.0 μCi of [*methyl*-^{3}H]thymidine (47 Ci/mmol; Amersham, Arlington Heights, Ill.) in 25 μl of RPMI 1640 was added per well, and the incorporation of [^{3}H]thymidine was quantified.

Statistics. The two-tailed unpaired Student *t* test, the Fisher exact test, and the Mann-Whitney U test were employed for analysis with the Statview IV (Abacus, Berkeley, Calif.) software program.

RESULTS

Characterization of the vaccine candidate preparations. A silver-stained gel of the four vaccine candidates is shown in Fig. 1. The preparations extracted with the SDS, OGP, and Z3-14 detergents showed predominantly a single band corresponding to the MOMP (40 kDa). In the COMC preparation, the MOMP, a doublet corresponding to the 60-kDa cysteine-rich protein (crp), and a band at 28 kDa were the main components observed. In addition, other bands with molecular masses of 100 kDa or higher were noted in the COMC lane.

Immune response to the vaccine preparations. Overall there was a high antibody response in the serum and vaginal washes for the four preparations used as immunogens. In Table 1, the data collected the day before the intrabursal challenge are shown. In general, the highest antibody serum levels to EB were observed in the mice inoculated with OGP-MOMP, while the lowest antibody response was obtained in the animals immunized with SDS-MOMP. Total antichlamydial serum IgG levels ranged from a titer of 51,200 in the animals immunized with OGP-MOMP to a titer of 12,800 in the mice immunized with SDS-MOMP. The distribution of antibody titer among the different IgG subclasses was similar among the four preparations, with the exception that the IgG2a levels obtained with SDS-MOMP and Z3-14–MOMP (100 and 200, respectively) were lower than those obtained with OGP-MOMP and COMC (6,400 and 6,500, respectively). The IgG1 serum levels were within 1 dilution for the four vaccine candidates. Only the control mice immunized intranasally with *C. trachomatis* EB

TABLE 1. Humoral and lymphoproliferative immune responses in mice the day before challenge

Group	*C. trachomatis* MoPn EB-specific serum ELISA antibody titer for:						Serum neutralizing titer	Vaginal wash ELISA antibody titer for:		Mean T-cell proliferative response (cpm, 10^3) ± 1 SE[a]			
	Whole IgG	IgG1	IgG2a	IgG2b	IgG3	IgA		IgG	IgA	EB[b]	Ovalbumin[c]	ConA[c]	None[d]
SDS-MOMP	12,800	6,400	100	1,600	1,600	200	200	64	16	18.3[e] ± 1.9	0.3 ± 0.1	129.7 ± 5.3	0.3 ± 0.05
OGP-MOMP	51,200	12,800	6,400	6,400	3,200	3,200	1,100	64	16	NT[f]	NT	NT	NT
Z3-14–MOMP	25,600	6,400	200	6,400	6,400	1,600	1,000	64	16	NT	NT	NT	NT
COMC	32,000	6,800	6,500	6,400	1,800	4,800	1,075	128	128	58.2[e] ± 3.7	2.4 ± 0.5	28.0 ± 1.1	1.5 ± 0.06
Ovalbumin	<100	<100	<100	<100	<100	<100	0	<2	<2	6.9 ± 1.4	9.2 ± 1.3	36.5 ± 2.3	0.45 ± 0.06
C. trachomatis MoPn	25,600	3,200	6,400	6,400	6,400	3,200	1,200	72	256	43.6[e] ± 4.1	0.2 ± 0.02	79.0 ± 6.7	0.2 ± 0.05
Sham immunized, nonchallenged	<100	<100	<100	<100	<100	<100	0	<2	<2	1.1 ± 0.2	0.4 ± 0.04	74.9 ± 12.8	0.07 ± 0.02

[a] Results are given for triplicate cultures. Data correspond to one of the experiments which was representative of two separate experiments.
[b] UV-inactivated *C. trachomatis* MoPn EBs were added at a ratio of 10:1 to accessory cells for antigen presentation.
[c] ConA and ovalbumin were each added at a concentration of 5 μg/ml.
[d] Only medium was added.
[e] Significant by the Student *t* test ($P < 0.05$) compared with the counts per minute for the ovalbumin-inoculated or sham-immunized, nonchallenged mice.
[f] NT, not tested.

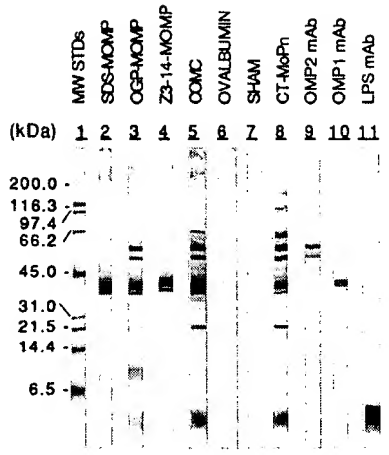

FIG. 2. Western blot of sera from immunized and control animals and monoclonal antibodies probed against *C. trachomatis* MoPn EB. Lane 8, sera from mice inoculated intranasally with *C. trachomatis* MoPn EB; lanes 9 to 11, monoclonal antibodies (mAb) to the 60-kDa crp, MOMP, and LPS, respectively. MW STDs, molecular weight standards.

had a slightly lower IgG1 level. IgA serum levels were lowest in the mice inoculated with SDS-MOMP and highest in the animals inoculated with COMC. Mice immunized with SDS-MOMP had the lowest neutralizing antibody titer in serum (200), while those immunized with the other three preparations had titers ranging from 1,000 to 1,100, similar to the 1,200 titer detected in the positive controls immunized intranasally with EB.

To further define the specificity of the antibody response, serum samples were tested by Western blotting against *C. trachomatis* EB. As shown in Fig. 2, the serum of the mice inoculated with SDS-MOMP and Z3-14–MOMP reacted mainly with MOMP. On the other hand, the serum from the mice immunized with OGP-MOMP and with the COMC reacted with MOMP, the 60-kDa crp, a 28-kDa component, and lipopolysaccharide (LPS). A distinct band was also observed at 10.5 kDa in the sera from the mice inoculated with the OGP-MOMP. The sera from the mice immunized with the COMC were the only serum samples that reacted with the affinity-purified 60-kDa hsp (data not shown).

The results of reacting the serum collected the day before challenge and 45 days after challenge against synthetic octameric peptides corresponding to the amino acid sequence of the *C. trachomatis* MoPn MOMP are shown in Fig. 3. Overall, the broadest antibody response before challenge to the four variable domains (VDs) was obtained with the sera from the mice immunized with the COMC and the Z3-14–MOMP preparations. Serum collected the day before the challenge from animals immunized with SDS-MOMP reacted only with VD1. Antibodies from mice inoculated with OGP-MOMP weakly reacted against VD1 and VD2. On the other hand, serum from mice immunized with the Z3-14–MOMP and COMC had antibodies that reacted against the four VDs, as did the control immunized with *C. trachomatis* MoPn EB. The consensus amino acid sequences recognized by most of the sera included TGD and ASR in VD1, VGL in VD2, VGQ in VD3, and TISG in VD4.

Serum collected 45 days after the genital challenge showed, in general, an amnanestic response to the VD that originally reacted on the day before the challenge, for all the vaccine preparations. No significant reactivity to the VD4 region was observed with the sera from the mice immunized with the SDS-MOMP, and only a very weak response was observed in those inoculated with the OGP-MOMP preparation. Control mice immunized with ovalbumin showed a minimal antibody response to VD1 and VD2 following the intrabursal challenge, with no significant increase in the antibody levels to VD3 or VD4. The COMC-inoculated group and the control group immunized intranasally with EB had a strong antibody response to the four VDs on the day before the challenge, and no significant change in antibody levels was obtained following the challenge.

Antibodies in the genital tract were measured by ELISA following washings of the vagina twice with 20 μl of PBS and a further 2× dilution before testing (Table 1). IgG-specific chlamydial antibodies were detected in the vaginas of all the four groups of immunized mice, with titers ranging from 64 to 128. The IgA titer of the three experimental groups immunized with purified MOMP was 16, while the IgA titer in the mice immunized with COMC was 128. The control mice inoculated intranasally with *C. trachomatis* EB had a vaginal IgA titer of 256.

T-cell response was determined in the groups of mice immunized with SDS-MOMP and with COMC (Table 1). With EB as the antigenic stimulus, a significant proliferative response was observed in the two groups that closely paralleled the response obtained with the control mice inoculated intranasally with *C. trachomatis* MoPn EB. For example, the stimulation index (cpm of the sample stimulated with EB/cpm of the sample stimulated with medium) was 60 and 39 for the mice immunized with the SDS-MOMP and the COMC preparations, respectively, and 14 for the control mice inoculated with ovalbumin. The mice immunized intranasally with EB had a stimulation index of 215.

The controls inoculated with ovalbumin and the group of mice that was sham immunized and not challenged had no chlamydia-specific antibodies and no significant T-cell proliferative response to the EB on the day before the challenge (Table 1).

Course of the infection following the intrabursal challenge. The results of the vaginal cultures obtained at weekly intervals following the intrabursal challenge are shown in Table 2. Overall, the most significant level of protection was observed in the animals immunized with COMC. In the control group inoculated with ovalbumin, 83% (40 of 48) of the mice had a positive culture during the 6 weeks of observation, while only 25% (4 of 20) of the animals immunized with COMC shed *C. trachomatis* vaginally ($P < 0.05$). Furthermore, the ovalbumin-inoculated mice had positive cultures up to 5 weeks following the challenge, while the group immunized with COMC had positive cultures only during the first 2-week postchallenge. In addition, the ovalbumin-inoculated mice shed a significantly higher number of infectious particles than did the COMC-immunized group.

Of the three groups immunized with SDS-MOMP, OGP-MOMP and Z3-14–MOMP, there were no significant differences compared with the ovalbumin-inoculated group regarding the total number of mice shedding following the challenge. The length of the shedding was, however, shorter in these three groups—3 to 4 versus 5 weeks—and the number of IFU for each mouse was lower in the group immunized with OGP-MOMP for the cultures collected during the 3rd week.

As expected, the positive controls immunized intranasally with *C. trachomatis* MoPn EB had very strong protection. Only 3% (1 of 35) of the animals shed vaginally and only during the 1st week postchallenge. In addition, the number of IFU was significantly lower. All the mice in the sham-immunized, nonchallenged group remained negative throughout the experiment.

Fertility studies. The results of the fertility studies are shown in Table 3. Of the control sham-immunized, nonchallenged

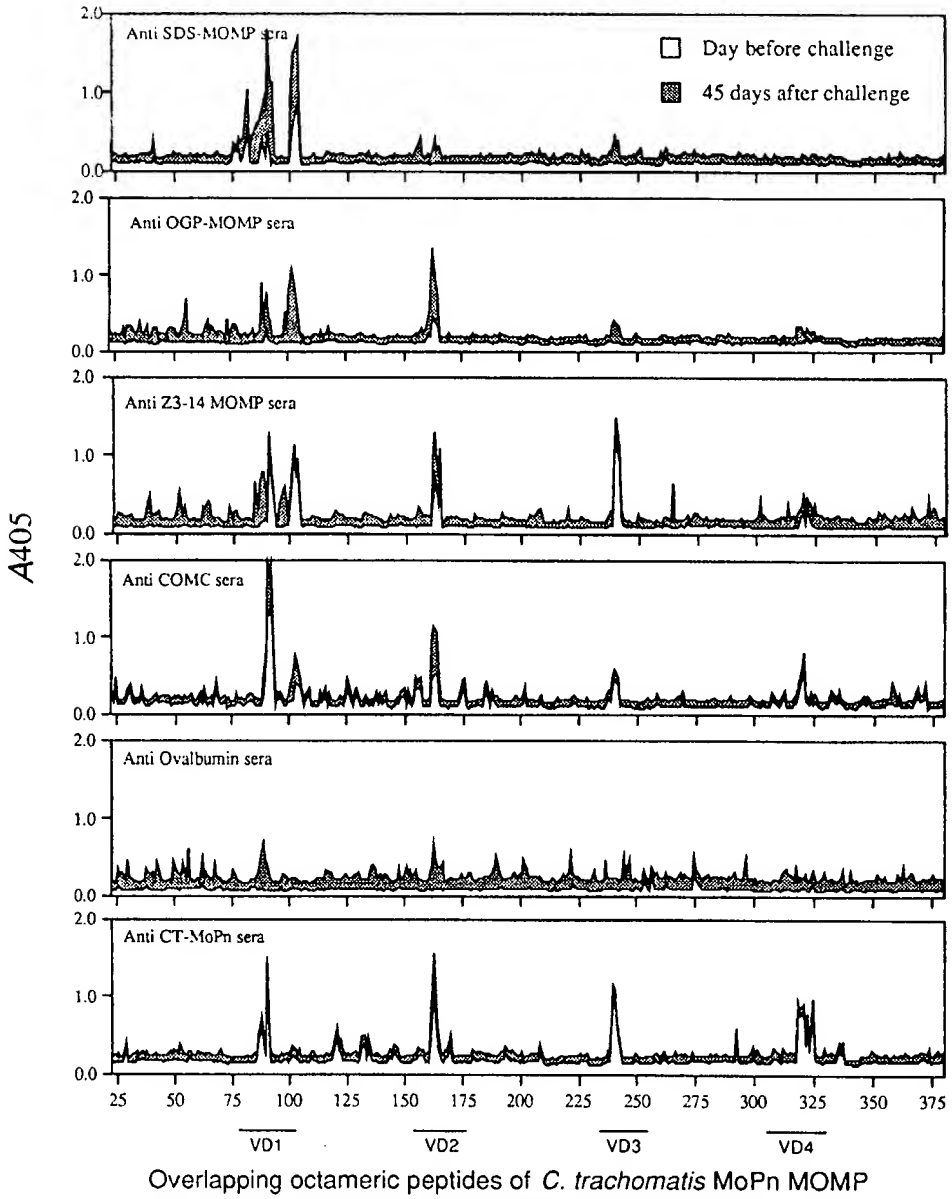

FIG. 3. Reactivity to octameric synthetic peptides in sera collected from different groups of mice on the day before the genital challenge and 45 days after the challenge.

mice, 81% (17 of 21) were bilaterally fertile in contrast to the ovalbumin-inoculated, *C. trachomatis*-challenged group, in which only 23% (11 of 48) of the mice were bilaterally fertile ($P < 0.05$). Mice immunized intranasally with the *C. trachomatis* MoPn EB were protected, as shown by the fact that 94% (33 of 35) of the animals had bilateral pregnancies. On the other hand, of the four groups inoculated with *C. trachomatis* acellular vaccines, only those immunized with COMC had a bilateral fertility rate of 70% (14 of 20), which was not statistically significant different from the rate for the control sham-immunized, nonchallenged group ($P > 0.05$). The groups immunized with SDS-, OGP-, and Z3-14–MOMP had fertility rates that were significantly lower than that of the control group ($P < 0.05$).

We also calculated the average number of embryos per mouse for each group of animals. As shown in Table 3, the average number of embryos was 5.9 in the control sham-immunized, nonchallenged group and 6.4 and 5.1 in the *C. trachomatis* MoPn EB- and COMC-immunized animals, respectively ($P > 0.05$). On the other hand, a significant decrease ($P < 0.05$) in the number of embryos was observed in the three groups immunized with SDS-, OGP- and Z3-14–MOMP.

DISCUSSION

Attempts to produce a vaccine to protect against *C. trachomatis* ocular infections were initiated several decades ago (17). Based on trials in humans and monkeys, some investigators

TABLE 2. Results of vaginal cultures

Group	Mean $\log_{10}$ *C. trachomatis* MoPn IFU shed ± 1 SE (% positive mice in group) for week:					Total no. of mice that shed in the 6 weeks/total in group (% positive)
	1	2	3	4	5	
SDS-MOMP	2.94 ± 2.81 (30)	3.44 ± 3.35 (50)	2.02 ± 2.0 (20)	1 ± 0 (10)	0 (0)	6/10 (60)
OGP-MOMP	4.14 ± 3.98 (30)	3.96 ± 3.73 (89)	0.78 ± 0.78[a] (22)	0 (0)	0 (0)	8/9 (89)
Z3-14-MOMP	4.31 ± 4.02 (40)	4.24 ± 3.89 (80)	3.39 ± 3.37 (50)	0 (0)	0 (0)	8/10 (80)
COMC	3.40 ± 3.40[a] (5)[b]	1.91 ± 1.91[a] (15)[b]	0.03 ± 0[a] (5)[b]	0 (0)	0 (0)	4/20 (25)[b]
Ovalbumin	4.10 ± 3.26 (27)	4.21 ± 2.76 (67)	3.8 ± 3.58 (50)	3.41 ± 3.32 (23)	1 ± 1 (2)	40/48 (83.3)
C. trachomatis MoPn	2.94 ± 2.94[a] (3)[b]	0[a] (0)[b]	0[a] (0)[b]	0[a] (0)[b]	0 (0)	1/35 (3)[b]
Sham-immunized, nonchallenged	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0/21 (0)

[a] Significant by the Mann-Whitney U test ($P < 0.05$) compared with values for the ovalbumin-inoculated mice.
[b] Significant by the Fisher exact test ($P < 0.05$) compared with values for the ovalbumin-inoculated mice.

reported that individuals vaccinated with the whole organism were protected when subsequently challenged, while others developed worse symptoms than those occurring in the nonvaccinated controls (17). The implication of these observations was that most likely, a component present in the whole organism predisposed the individual to a hypersensitivity reaction upon reexposure. Based on this assumption, recent attempts have been made at producing acellular vaccines with only purified chlamydial antigens. MOMP appears to be the best candidate for an acellular vaccine, since it is surface exposed, accounts for almost 60% of the outer membrane protein mass, and is strongly antigenic (2, 3, 8, 9, 41). Stephens et al. (34), based on the comparison of the DNA and amino acid sequence of the L2, B, and C *C. trachomatis* serovars, showed that this protein has four VDs interspersed with five constant domains. The same overall molecular structure has now been confirmed for all human strains and the MoPn biovar (13, 14, 47). The four VDs identified in MOMP elicit neutralizing antibodies with serovar, subgroup, and subspecies specificities, while T-cell epitopes have been mapped throughout the molecule (20, 23, 25, 26, 35).

However, efforts to vaccinate with purified *C. trachomatis* MOMP or peptides corresponding to the variable domains of MOMP have so far resulted in very limited success. For example, Taylor et al. (39) immunized monkeys with SDS-extracted MOMP and cholera toxin as an adjuvant in an attempt to induce protection against an ocular challenge. They observed that although there was a transient decrease in the inflammatory response, no reduction in the duration or intensity of the infection occurred. Tuffrey et al. (40) immunized mice with a recombinant serovar L1 MOMP expressed in *Escherichia coli* and purified with SDS and subsequently challenged the animals in the ovarian bursae or intravaginally with the human serovar F. Overall, although they observed in some groups a decrease in the severity of the salpingitis and in others a decrease in shedding, they were not able to demonstrate protection against infertility. Similarly, Batteiger et al. (1) immunized guinea pigs with the SDS-extracted MOMP of the *Chlamydia psittaci* agent of guinea pig inclusion conjunctivitis (GPIC) and observed no difference with the control nonimmunized animals following an intravaginal challenge. Also, Su et al. (36) inoculated mice with a synthetic oligopeptide corresponding to the *C. trachomatis* MOMP, containing a common T-helper and neutralizable B-cell epitopes, and detected no differences between the immunized and the control mice regarding colonization, shedding, or duration of the infection.

These investigators have proposed that the inability of these preparations to induce protective immunity was most likely due to the loss of conformational epitopes in MOMP. Alternatively, the need for additional chlamydial antigens and the inadequacy of the immunization protocols to induce a protective response in the mucosa were considered possible explanations for the failure of these approaches. In this respect, it is interesting that up to now, the most successful results with an acellular chlamydial vaccine were reported by Tan et al. (38), who used an outer membrane preparation from *C. psittaci*. This preparation was found by electron microscopy to maintain the overall structure of an EB and by electrophoresis to contain the MOMP, the 60-kDa crp, and the LPS. When Tan et al. (38) immunized sheep with this preparation, they obtained protection against abortion following a subcutaneous challenge with the infectious organisms. On the other hand, Batteiger et al. (1) immunized guinea pigs with an outer membrane preparation, obtained like one of our MOMP immunogens with Sarkosyl and OGP, challenged the animals intravaginally with the *C. psitacci* GPIC, and observed a decrease in the quantity of shedding but not in the duration of the infection. This preparation was determined to contain mainly MOMP in addition to other components, including proteins at 84, 72, 61, 47, 35, and 33 kDa and LPS.

Here, we have followed a protocol similar to the one reported by Tan et al. (38) to prepare an acellular vaccine from *C. trachomatis* MoPn and have shown that immunization with this preparation protects mice against infection and infertility following an intrabursal challenge. In contrast, purified *C. trachomatis* MoPn MOMP extracted using three different protocols with an anionic (SDS), a nonionic (OGP) and a zwitterionic (Z3-14) detergent failed for the most part to protect the mice against a genital challenge. The fact that the COMC was able to protect, while the purified MOMP preparations were

TABLE 3. Results of fertility studies

Group	No. of mice bilaterally fertile/total (% fertility)	Mean no. of embryos/mouse ± 1 SD
SDS-MOMP	1/9 (11)[a]	2.0 ± 1.3[b]
OGP-MOMP	3/9 (33)[a]	3.9 ± 1.5[b]
Z3-14-MOMP	1/10 (10)[a]	1.7 ± 1.8[b]
COMC	14/20 (70)	5.1 ± 3.0
Ovalbumin	11/48 (23)[a]	3.3 ± 2.7[b]
C. trachomatis MoPn	33/35 (94)	6.4 ± 2.9
Sham-immunized, nonchallenged	17/21 (81)	5.9 ± 2.6

[a] Significant by the Fisher exact test ($P < 0.05$) compared with values for the sham-immunized, nonchallenged mice.
[b] Significant by the Student t test ($P < 0.05$) compared with values for the sham-immunized, nonchallenged mice.

not, could be ascribed to several factors, including the presence of antigens other than the MOMP in the COMC and the different immunization protocol used with this preparation (38). Therefore, the possibility that a detergent-extracted MOMP preparation in conjunction with the immunization protocol described for COMC may be able to protect cannot be completely excluded until such a protocol is tested. In our opinion, however, the ability of the COMC to protect is most likely due to the preservation of conformational epitopes or to the accessibility of the protective epitopes on MOMP to the immune cells. Here, the use of glutaraldehyde as outlined by Tan et al. (38) may have helped to fix the structure of the purified COMC and thus to maintain the native conformational epitopes of the EB. In the purified MOMP preparations, the conformational protective epitopes may be destroyed during the extraction procedure or they may be blocked by the detergent so that they are not accessible to the immune system. In this respect, we have recently shown that in the mouse model, intraperitoneal inoculation with a neutralizing IgA monoclonal antibody to a conformational epitope of the *C. trachomatis* MoPn MOMP resulted in protection against a genital challenge (23). In contrast, another IgA monoclonal antibody to a linear epitope of the MOMP had only minimal protective ability. Finally, Whittum-Hudson et al. (46) have recently reported that oral immunization with an anti-idiotypic antibody to the exoglycolipid antigen of *C. trachomatis* resulted in a limited protection against an ocular challenge. Thus, at this point, the question of which chlamydial components may be used as a vaccine to protect against infection and/or disease remains open.

The immunological mechanisms involved in protection against a chlamydial genital infection are poorly understood. Humoral and cell immune responses may be critical at different stages of the infection (41). For example, antibodies may inhibit the entry of EB by blocking cellular receptors or by modifying the EB surface charge and hydrophobicity. The genital mucosa could respond to an infection producing secretory IgA antibodies that block the pathogen at the site of entry. EB could also be opsonized by IgG or be killed by an antibody-dependent cellular cytotoxic attack. Furthermore, $CD4^+$ and $CD8^+$ T cells, phagocytes, and cytokines may clear the organism at later stages of the infection. In this respect, Morrison et al. (19), using gene knockout mice, demonstrated that major histocompatibility complex class II-restricted T-cell responses are required for protective immunity against a genital infection. In their experiments, $\beta 2m^{-/-}$ mice resolved a genital infection like the control immunocompetent animals, while $CD4^{-/-}$ animals showed a delayed resolution and class $II^{-/-}$ mice were unable to control the infection. In addition, these authors reported that all animals that recovered from the primary infection had decreased shedding upon reinfection. These findings support the observations reported by Igietseme et al. (18) showing that persistently infected T-cell deficient nude mice could resolve an infection following the adoptive transfer of a $CD4^+$ Th-1 T-cell clone that secreted high levels of interleukin-2, tumor necrosis factor, and gamma interferon. Thus, $CD4^+$ T cells appear to play a significant role in controlling the infection. On the other hand, the role that $CD8^+$ T cells may have in a chlamydial infection remains controversial, with both in vitro and in vivo experiments from several laboratories yielding contradictory results (19, 24, 33).

We detected significant levels of IgA antibodies in the genital tracts of the mice immunized with the COMC preparation and in those of the control group inoculated intranasally with EB, the two groups that were protected against infection and disease. These results further substantiate the observation reported by Brunham et al. (5) and our results with the anti-MOMP IgA monoclonal antibody (23) and suggest that in a genital infection, mucosal humoral immunity may be sufficient to protect against infection. Furthermore, if an intracellular pathogen such as *Chlamydia* is to be blocked before it can infect a cell and thus produce sterilizing immunity, a vaccine will have to induce antibodies or other factors that are at the site of entry. Antibodies can neutralize infectivity on mucosal surfaces before a pathogen infects a cell, while the effector mechanisms of cell-mediated immunity can operate only after cells are infected. This may be different than the requirements to control an already established infection, in which the cell-mediated immune response may be sufficient to eradicate the organism (28, 29). In addition, in the case of a chlamydial genital infection, protection against long-term disease, e.g., infertility, ectopic pregnancy or chronic abdominal pain, may be achieved with a vaccine that does not necessarily protect against the infection. This would not be surprising, since most human vaccines protect against disease but not against infection (30).

We have analyzed the response to the different MOMP preparations, using octameric synthetic peptides. This technique is quite powerful, since it allows the determination at the molecular level of the antigenic sites able to induce a humoral immune response. However, an obvious shortcoming of this approach is that it does not detect conformational epitopes. In spite of this shortcoming, our results show that the four preparations tested elicited different immune responses. Overall, reactivity to VD1 was observed in all the groups of animals, although it was very weak in those immunized with OGP-MOMP, and reactivity to VD2 was found in all groups except those inoculated with SDS-MOMP. Antibodies to all four VDs were present only in the control mice that were intranasally immunized with EB and in the groups that were inoculated with the Z3-14–MOMP and the COMC preparations. In this respect, Batteiger et al. (1), using recombinant fragments corresponding to the four VDs of the *C. psittaci* GPIC MOMP, tested the sera from guinea pigs immunized with SDS-MOMP and OGP-MOMP and from guinea pigs that had recovered from a genital infection and failed to observe significant differences in reactivity to any of the recombinant peptides corresponding to the four VDs. These differences with our results may be due to the types of methodologies used to detect antibodies to the VD, the use of octameric synthetic peptides versus recombinant peptide fragments, or to the differences in the immune responses elicited by the immunogens in mice in contrast to those in guinea pigs. Also, Su et al. (35) used the *C. trachomatis* MOMP from serovar A extracted with SDS to immunize six inbred strains of mice, with different *H-2* haplotypes, and using synthetic peptides of 25 residues, they observed that VD1 and VD4 were the immunodominant regions. Again, this difference with our results, in which we did not detect reactivity to VD4 with the serum from the animals inoculated with the SDS-MOMP, may represent a true difference in the immunological response of the particular strain of mice or a technical artifact due to the methodologies that were utilized to detect the antibodies.

In conclusion, here we have shown for the first time that an acellular vaccine containing mainly the *C. trachomatis* MOMP, the 60-kDa crp, and LPS can protect against infection and against disease, e.g., infertility, following a genital challenge. This observation clearly establishes that inoculation with the whole organism is not necessary in order to protect against a *C. trachomatis* genital infection. A caveat with this model may be that the animals were challenged intrabursally. Thus, it could be argued that this immunization protocol may not protect

against an intravaginal challenge, the natural route of infection, since the defense mechanisms operating in the lower and upper genital tract may be different. In our opinion, however, by inoculating intrabursally we are providing a more stringent proof of protection, since we are challenging the mice directly at the site where disease occurs, e.g., the oviduct, while bypassing sites such as the cervix, where most likely our immunization protocol will induce additional protective responses. Eventually, so that infertility and ectopic pregnancy can be protected against, the fallopian tube is the site at which the vaccine will have to block the spread of the infection. The ability to protect against an intravaginal challenge will nevertheless have to be experimentally tested before final conclusions can be reached. In addition, further work is necessary in order to identify and characterize the specific antigenic sites capable of inducing protection and the humoral and cellular components of the immune response that effect resistance against the infection.

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REFERENCES

1. **Batteiger, B. E., R. G. Rank, P. M. Bavoil, and L. S. F. Soderberg.** 1993. Partial protection against genital reinfection by immunization of guinea-pigs with isolated outer-membrane proteins of the chlamydial agent of guinea-pig inclusion conjunctivitis. J. Gen. Microbiol. **139**:2965–2972.
2. **Bavoil, P. M., R.-C. Hsia, and R. G. Rank.** 1996. Prospects for a vaccine against *Chlamydia* genital disease I. Microbiology and pathogenesis. Bull. Inst. Pasteur **94**:5–54.
3. **Bavoil, P. M., A. Ohlin, and J. Schachter.** 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. **44**:479–485.
4. **Brunham, R. C., B. Binns, J. McDowell, and M. Paraskevas.** 1986. *Chlamydia trachomatis* infection in women with ectopic pregnancy. Obstet. Gynecol. **67**:722–726.
5. **Brunham, R. C., C. C. Kuo, L. Cles, and K. K. Holmes.** 1983. Correlation of host immune response with quantitative recovery of *Chlamydia trachomatis* from the human endocervix. Infect. Immun. **39**:1491–1494.
6. **Brunham, R. C., I. W. Maclean, B. Binns, and R. W. Peeling.** 1985. *Chlamydia trachomatis*, its role in tubal infertility. J. Infect. Dis. **152**:1275–1282.
7. **Brunham, R. C., R. Peeling, I. Maclean, J. McDowell, K. Persson, and S. Osser.** 1987. Postabortal *Chlamydia trachomatis* salpingitis: correlating risk with antigen-specific serological responses and with neutralization. J. Infect. Dis. **155**:749–755.
8. **Caldwell, H. D., J. Kromhout, and J. Schachter.** 1981. Purification and characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. **31**:1161–1176.
9. **Caldwell, H. D., and J. Schachter.** 1982. Antigenic analysis of the major outer membrane protein. Infect Immun. **35**:1024–1031.
10. **Cates, W., R. T. Rolfs, and S. O. Aral.** 1990. Sexually transmitted diseases, pelvic inflammatory disease and infertility: an epidemiologic update. Epidemiol. Rev. **12**:199–220.
11. **de la Maza, L. M., S. Pal, A. Khamesipour, and E. M. Peterson.** 1994. Intravaginal inoculation of mice with the *Chlamydia trachomatis* mouse pneumonitis biovar results in infertility. Infect. Immun. **62**:2094–2097.
12. **de la Maza, M. A., and L. M. de la Maza.** 1995. A new computer model for estimating the impact of vaccination protocols and its application to the study of *Chlamydia trachomatis* genital infections. Vaccine **13**:119–127.
13. **Fielder, T. J., S. Pal, E. M. Peterson, and L. M. de la Maza.** 1991. Sequence of the gene encoding the major outer membrane protein of the mouse pneumonitis biovar of *Chlamydia trachomatis*. Gene **106**:137–138.
14. **Fitch, W. M., E. M. Peterson, and L. M. de la Maza.** 1993. Phylogenetic analysis of the outer membrane protein genes of *Chlamydiae* and its implications for vaccine development. Mol. Biol. Evol. **10**:892–913.
15. **Geysen, H. M., S. J. Rodda, T. J. Mason, G. Tribbick, and P. Schoofs.** 1987. Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods **102**:259–274.
16. **Grayston, J. T., and S. P. Wang.** 1975. New knowledge of *Chlamydiae* and the diseases they cause. J. Infect. Dis. **132**:87–105.
17. **Grayston, J. T., and S. P. Wang.** 1978. The potential for vaccine against infection of the genital tract with *Chlamydia trachomatis*. Sex. Transm. Dis. **5**:73–77.
18. **Igietseme, J. U., K. H. Ramsey, D. M. Magee, D. M. Williams, T. J. Kincy, and R. G. Rank.** 1993. Resolution of murine chlamydial genital infection by the adoptive transfer of a biovar-specific Th1 lymphocyte clone. Reg. Immunol. **5**:317–324.
19. **Morrison, R. D., K. Feilzer, and D. B. Tumas.** 1995. Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. Infect. Immun. **63**:4661–4668.
20. **Pal, S., X. Cheng, E. M. Peterson, and L. M. de la Maza.** 1993. Mapping of a surface exposed B-cell epitope to the variable sequent 3 of the major outer membrane protein of *Chlamydia trachomatis*. J. Gen. Microbiol. **139**:1565–1570.
21. **Pal, S., T. J. Fielder, E. M. Peterson, and L. M. de la Maza.** 1993. Analysis of the immune response in mice following intrauterine infection with the *Chlamydia trachomatis* mouse pneumonitis biovar. Infect. Immun. **61**:772–776.
22. **Pal, S., T. J. Fielder, E. M. Peterson, and L. M. de la Maza.** 1994. Protection against infertility in a BALB/c mouse salpingitis model by intranasal immunization with the mouse pneumonitis biovar of *Chlamydia trachomatis*. Infect. Immun. **62**:3354–3362.
23. **Pal, S., I. Theodor, E. M. Peterson, and L. M. de la Maza.** 1997. Monoclonal immunoglobulin A antibody to the major outer membrane protein of the *Chlamydia trachomatis* mouse pneumonitis biovar protects mice against a chlamydial genital challenge. Vaccine **15**:575–582.
24. **Pavia, C. S., and J. Schachter.** 1983. Failure to detect cell-mediated cytotoxicity against *Chlamydia trachomatis*-infected cells. Infect. Immun. **39**: 1271–1274.
25. **Peterson, E. M., X. Cheng, B. Markoff, T. J. Fielder, and L. M. de la Maza.** 1991. Functional and structural mapping of *Chlamydia trachomatis* species-specific major outer membrane epitopes by use of neutralizing monoclonal antibodies. Infect. Immun. **59**:4147–4153.
26. **Peterson, E. M., G. Zhong, E. Carlson, and L. M. de la Maza.** 1988. Protective role of magnesium in the neutralization by antibodies of *Chlamydia trachomatis* infectivity. Infect. Immun. **56**:885–891.
27. **Punnonen, R., P. Terho, V. Nikkanen, and O. Meuraman.** 1979. Chlamydial serology in infertile women by immunofluorescence. Fertil. Steril. **31**:656–659.
28. **Ramsey, K. H., and R. G. Rank.** 1991. Resolution of chlamydial genital infection with antigen specific T-lymphocyte lines. Infect. Immun. **59**:925–931.
29. **Ramsey, K. H., L. S. F. Soderberger, and R. G. Rank.** 1988. Resolution of chlamydial genital infection in B-cell-deficient mice and immunity to reinfection. Infect. Immun. **56**:1320–1325.
30. **Roizman, B.** 1991. Introduction: objectives of herpes simplex virus vaccines seen from historical perspective. Rev. Infect. Dis. **13**:S892–S894.
31. **Schachter, J., and C. Dawson.** 1978. Human chlamydial infections. PSG Publishing Company, Inc., Littleton, Mass.
32. **Schagger, H., and G. Von Jagow.** 1987. Tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis for the separation of protein range 1 to 100 kDa. Anal. Biochem. **166**:368–379.
33. **Starnbach, M. N., M. J. Bevan, and M. F. Lampe.** 1994. Protective cytotoxic T lymphocytes are induced during murine infection with *Chlamydia trachomatis*. J. Immunol. **153**:5183–5189.
34. **Stephens, R. S., R. Sanchez-Pescador, E. A. Wagar, C. Inouye, and M. S. Urdea.** 1987. Diversity of *Chlamydia trachomatis* major outer membrane protein genes. J. Bacteriol. **169**:3879–3885.
35. **Su, H., R. P. Morrison, N. G. Watkins, and H. D. Caldwell.** 1990. Identification and characterization of T helper cell epitopes of the major outer membrane protein of *Chlamydia trachomatis*. J. Exp. Med. **172**:203–212.
36. **Su, H., M. Parnell, and H. D. Caldwell.** 1995. Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of *Chlamydia trachomatis* genital tract infection: serum neutralizing IgG antibodies do not protect against chlamydial genital infection. Vaccine **13**:1023–1031.
37. **Swenson, C. E., and J. Schachter.** 1984. Infertility as a consequence of chlamydial infection of the upper genital tract in female mice. Sex. Transm. Dis. **11**:64–67.
38. **Tan, T.-W., A. J. Herring, I. E. Anderson, and G. E. Jones.** 1990. Protection of sheep against *Chlamydia psittaci* infection with a subcellular vaccine containing the major outer membrane protein. Infect. Immun. **58**:3101–3108.
39. **Taylor, H., J. Whittum-Hudson, J. Schachter, H. D. Caldwell, and R. A. Prendergast.** Oral immunization with chlamydial outer membrane protein (MOMP). 1988. Invest. Ophthalmol. Visual Sci. **29**:1847–1853.
40. **Tuffrey, M., F. Alexander, W. Conlan, C. Woods, and M. Ward.** 1992. Heterotypic protection of mice against chlamydial salpingitis and colonization of the lower genital tract with a human serovar F isolate of *Chlamydia trachomatis* by prior immunization with recombinant serovar L1 major outer-membrane protein. J. Gen. Microbiol. **138**:1707–1715.
41. **Ward, M. E.** 1992. Chlamydial vaccines—future trends. J. Infect. **25**:S11–S26.
42. **Washington, A. E., and P. Katz.** 1991. Cost of and payment source for pelvic inflammatory disease. JAMA **266**:2565–2569.

43. **Washington, A. E., S. O. Aral, P. Wolner-Hanssen, D. A. Grimes, and K. K. Holmes.** 1991. Assessing risk for pelvic inflamatory disease and its sequelae. JAMA **266**:2581–2586.
44. **Westrom, L., R. Joesoef, G. Reynolds, A. Hagdu, and S. E. Thompson.** 1992. Pelvic inflammatory disease and fertility: a cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopy. Sex. Transm. Dis. **19**:185–192.
45. **Westrom, L., and P. A. Mardh.** 1990. Acute pelvic inflammatory disease (PID), p. 593–614. *In* K. K. Holmes, P. A. Mardh, P. F. Sparling, et al. (ed.), Sexually transmitted diseases. 2nd ed. McGraw-Hill, New York, N.Y.
46. **Whittum-Hudson, J. A., L.-L. An, W. M. Saltzman, R. A. Prendergast, and B. MacDonald.** 1996. Oral immunization with an anti-idiotypic antibody to the exoglycolipid antigen protects against experimental *Chlamydia trachomatis* infection. Nat. Med. **2**:116–121.
47. **Yuan, Y., Y. X. Zhang, N. G. Watkins, and H. D. Caldwell.** 1989. Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. Infect. Immun. **57**:1040–1049.

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Poliovirus Hybrids Expressing Neutralization Epitopes from Variable Domains I and IV of the Major Outer Membrane Protein of *Chlamydia trachomatis* Elicit Broadly Cross-Reactive *C. trachomatis*-Neutralizing Antibodies

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Trachoma and sexually transmitted diseases caused by *Chlamydia trachomatis* are major health problems worldwide. Epitopes from the variable domains of the major outer membrane protein are candidates for vaccine development. We have constructed hybrid polioviruses expressing sequences from major outer membrane protein variable domains I and IV. Antisera to the hybrids could, in combination, strongly neutralize 8 of the 12 *C. trachomatis* serovars most commonly associated with oculogenital infections and weakly neutralize the others.

Chlamydia trachomatis infects the epithelia of the conjunctivae and genital tract, causing blinding trachoma and a variety of sexually transmitted diseases (STDs) with sequelae that include pelvic inflammatory disease, ectopic pregnancies, and infertility (5, 20, 21, 28). The estimated cost of treating chlamydial STDs and their sequelae is over 4 billion dollars per year in the United States alone (28, 29). Trachoma, which is the leading cause of preventable blindness in developing nations, afflicts an estimated 600 million people worldwide (5). Development of an effective vaccine would be a valuable contribution to the control of chlamydial diseases. *C. trachomatis* isolates associated with trachoma and STDs can be immunotyped into 12 distinct serovars and two serogroups based on antigenic relatedness. Serovars A, C, H, I, J, and K are members of the C complex, whereas serovars B, Ba, D, E, F, and G are classified in the B and B-related complexes. Serovars A, B, Ba, and C are the causative agents of trachoma, while serovars D to K are primarily associated with STDs (5, 12, 20, 21).

The pathophysiology of chlamydial oculogenital diseases is thought to involve repeated infections and the generation of a pathologic hypersensitivity response to chlamydial antigen(s) (8, 9, 25, 26). Past attempts to develop whole-cell chlamydial vaccines by using killed elementary bodies (EBs) frequently potentiated disease by sensitizing vaccinees, and any protection observed in these studies was limited to the immunizing serovar (7, 8). Consequently, an effective chlamydial vaccine will probably have to be based on a subunit immunogen capable of inducing a strong protective immune response without sensitizing the vaccinee. Moreover, it would be advantageous if such a subunit immunogen were capable of inducing protection against multiple serovars and could target broadly cross-protective immunity to mucosal surfaces, since these are the sites colonized by chlamydiae.

The most promising candidate antigen for the development of a subunit vaccine capable of stimulating local antichlamydial neutralizing antibodies is the chlamydial major outer membrane protein (MOMP) (3). The primary sequence of MOMP is highly conserved among serovars with the exception of four symmetrically spaced variable domains (VDI to VDIV) that exhibit considerable interserovar sequence variation (30). Neutralizing monoclonal antibodies (MAbs) that differentiate *C. trachomatis* isolates into serogroups and serovars recognize the MOMP, and the binding sites of MOMP-specific neutralizing MAbs have been mapped to VDI, VDII, or VDIV, depending on the serovar (2, 4, 10, 18, 22, 31, 32). These antigenic sites represent important targets for the development of a subunit or synthetic vaccine against *C. trachomatis*.

We are interested in using recombinant poliovirus-MOMP hybrids as models for the development of a live attenuated vector-based chlamydial vaccine since such vectors could theoretically elicit antichlamydial neutralizing antibodies at mucosal surfaces. Live attenuated polio vaccine is usually administered orally, with the object of inducing enteric mucosal immunity, but has also been reported to be capable of inducing detectable antibody in vaginal and uterine secretions following oral administration (17). We have previously described (14) a hybrid type 1 poliovirus, PV1-Ct7, which expresses MOMP sequences corresponding to VDI of serovar A, including the epitope VAGLEK recognized by the serovar A-specific neutralizing MAb A-20 (2, 31, 32). We showed that PV1-Ct7 is highly immunogenic and can induce neutralizing antisera cross-reactive with serovars A and C. To better assess the potential of this hybrid to evoke cross-reactive neutralizing antibody responses, we have extended our studies on the neutralizing activity of antisera generated against PV1-Ct7 and the related hybrid PV1-Ct8 to include other C-complex serovars. We have additionally extended our studies to include the construction of an additional family of hybrids expressing MOMP VDIV sequences that contain the highly conserved neutralizing epitope LNPTIAG found in all serovars except K (in which the equivalent sequence is LNPTITG) (2, 30–32).

Hybrid polioviruses were constructed by using the mutagenesis cartridge strategy of Murray et al. (16). The construction of PV1-Ct7 and PV1-Ct8, which express serovar A MOMP VDI sequences (Fig. 1), has been described previously (14). To

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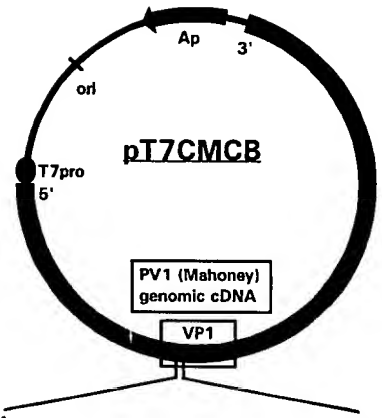
(A)



Sal I Hind III

nt 2753 g|TC GAC AAC CCA GCT TCC ACC ACC AAT AAG GAC A|AG CTT 2791
CAG CT| G TTG GGT CGA AGG TGG TGG TTA TTC CTG TTC GA|A

aa1094 V D N P A S T T N K D K L 1102

(B)

Clone	Sequence encoded (Chlamydial sequences underlined)	Viable virus recovered/ strain designation
pT7XLD	NPASTT NKD	Yes/PV1-XLD
pT7CtIVA1	NPASTTLNPTIAGAGDVKD	Yes/PV1-CtIVA
pT7CtIVB4	NPASTTLNPTIAGAGD	Yes/PV1-CtIVB
pT7CtIVC9	NPASTTLNPTIAGA KD	Yes/PV1-CtIVC
pT7Ct7	NPASTTSDVAGLEKDPVA	Yes/PV1-Ct7
pT7Ct8	NPASTTSDVAGLEKDP KD	Yes/PV1-Ct8

FIG. 1. Construction of poliovirus-chlamydia hybrids. By using a *SalI-Hin*dIII mutagenesis cartridge, the PV1-Mahoney cDNA clone pT7CMCB was modified to encode amino acid sequences, including the LNPTIAG epitope, from B-complex *C. trachomatis* MOMP VDIV. The sequence TTLNPTIAGAGDVK is conserved among serovars B, Ba, D, and E. (A) The mutagenesis cartridge spans poliovirus nucleotides (nt) 2759 to 2785, which encode poliovirus amino acids (aa) 1094 to 1102. The BC loop of VP1 comprises amino acids ASTTNKDKL, which are underlined. (B) The poliovirus-specific nucleotide sequence within the cartridge was replaced with synthetic oligonucleotides encoding three amino acid sequences from B-complex *C. trachomatis* MOMP VDIV, as underlined in the lower part of the figure. Viable virus was recovered, as described in the text, from all three clones. The serovar A MOMP VDI sequences expressed by PV1-Ct7 and PV1-Ct8 are shown for comparison; their construction has been described previously (14).

construct the VDIV hybrids, synthetic oligonucleotides coding for conserved amino acid sequences from MOMP VDIV of the *C. trachomatis* B-complex serovars B, Ba, D, and E were cloned into a full-length cDNA of PV1-Mahoney in place of sequences coding for amino acids within the BC loop of poliovirus capsid protein VP1 (Fig. 1). Hybrid viruses were prepared from these cDNAs as described previously (14, 16, 27) and were designated PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC (Fig. 1). In a single-step growth cycle, all were moderately impaired in comparison with PV1-XLD (data not shown), but all three hybrids grew sufficiently well to permit the easy preparation of virus stocks for further study.

The antigenic characteristics of PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC are shown in Table 1. Susceptibility of these viruses to neutralization by MAb DIII-A3 was examined. DIII-A3 is specific to *C. trachomatis* MOMP and recognizes the LNPTIAG epitope located in VDIV. It binds to intact EBs of serovars B, Ba, D, E, L1, L2, F, G, and L3 in a dot immunoblot and neutralizes their infectivity in vitro (2, 31, 32). PV1-CtIVA was extremely sensitive to DIII-A3, whereas PV1-CtIVB required approximately 100-times-greater concentrations of the MAb to achieve the same degree of neutralization. Neutralization of PV1-CtIVC by DIII-A3 was not detectable at the highest concentration tested. In contrast, all three hybrids were sensitive to neutralization by convalescent sera obtained 120 days postinfection from cynomolgus monkeys that had recovered from a primary cervical infection with *C. trachomatis* serovar D, although titers against PV1-CtIVC were about four-fold lower than titers against the other two VDIV hybrids. The VDIV hybrids clearly express at least one *C. trachomatis*-specific epitope in a conformation which is presumably similar to the native conformation. Interestingly, the epitope (or epitopes) is not expressed equivalently on each hybrid, as evidenced by the neutralization titers of MAb DIII-A3 and the convalescent sera. The DIII-A3 (LNPTIAG) epitope is highly accessible on PV1-CtIVA and is progressively less accessible on the surfaces of PV1-CtIVB and PV1-CtIVC. This may be due to the progressive C-terminal truncation of the VDIV sequences expressed or to changes in the conformation of the VDIV sequence that affect the surface accessibility of the epitope.

Rabbits were immunized with the VDIV hybrid viruses as described previously (14). Pools of antisera were tested by Western blotting (immunoblotting) against solubilized chlamydial EB proteins to determine the specificity of the anti-MOMP response (14). Chlamydial EBs were purified by density gradient centrifugation (3) from HeLa 229 cells infected with *C. trachomatis* serovar A (strain Har-13), B (strain TW-5), Ba (strain AP-2), C (strain TW-3), D (strain UW-3/Cx), E (strain Bour), F (strain IC-Cal-13), G (strain UW-57/Cx), H (strain UW-4/Cx), I (strain UW-12/Ur), J (strain UW-36/Cx), or K (strain UW/31/Cx). These 12 strains represent the chlamydial serovars commonly associated with trachoma or chlamydial STDs. As shown in Fig. 2, rabbit antisera raised against PV1-Ct7 reacted very strongly with the homotypic serovar A MOMP and less strongly with the MOMPs of serovars C, I, and J. Antisera raised against PV1-Ct8 showed a slightly different profile, reacting approximately equally with the MOMPs of serovars A, C, I, J, and K. In contrast, antisera raised against the VDIV hybrids reacted strongly with the MOMPs of all 15 serovars.

These results confirm species-wide conservation of at least some VDIV epitopes, and more limited conservation of some VDI epitopes, in the context of the denatured MOMP. Antigenic cross-reactivity occurs even in cases where the identified epitopes (VAGLEK or LNPTIAG) are not completely conserved. Thus, antisera to PV1-Ct7 and PV1-Ct8 recognize the serovar A VDI sequence TTSDVAGLEKDPVA and also the equivalent sequences from serovars C and J (TTSDVAGLQN DPTT; differences are underlined), and I (TTKDVAGLEND PVA). Similarly, antisera to PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC recognize sequences from VDIV of serovars B, Ba, D, E, F, G, C, A, H, I, and J (TTLNPTIAG) and of serovar K (TTLNPTITG). This finding indicates that the VDI and VDIV sequences expressed on the hybrids (Fig. 1) contain chlamydial epitopes in addition to VAGLEK and LNPTIAG. These additional epitopes are conserved between at least some serovars, and they can be effectively expressed by the poliovirus hybrids.

The specificities of the antisera for native MOMP were tested by dot blotting using viable EBs as the antigen (32). The cross-reactivity of the antichlamydial immune response elicited by the hybrids was not as great in the context of the native antigen. Antisera from rabbits immunized with PV1-Ct7 (rabbits 13 to 16) or PV1-Ct8 (rabbits 17 to 20) showed some

TABLE 1. Neutralization of poliovirus-MOMP hybrids by antichlamydial MAbs and sera

MAb or serum[a]	Neutralization titer[b] against:			
	PV1-CtIVA	PV1-CtIVB	PV1-CtIVC	PV1-XLD
DIII-A3	10,240	135	<20	<20
705				
Prebleed	<10	<10	<10	<10
Convalescent phase	299	423	92	<10
Anti-PV1	17,782	40,960	13,388	28,973

[a] MAb DIII-A3 was raised against *C. trachomatis* serovar D and is specific for the MOMP VDIV epitope LNPTIAG (2, 31, 32). Serum 705 was obtained from a monkey infected with *C. trachomatis* serovar D. Anti-PV1 is a PV1-specific rabbit pool.

[b] Reciprocal dilution of the serum or MAb giving a 50% endpoint in a neutralization assay versus 100 tissue culture 50% infective doses of virus conducted with Vero cells as described by Golding et al. (6). Assays were performed in microtiter plates, with eight replicates per sample per assay, and positive results were confirmed by repetition. Dilutions of DIII-A3 refer to a starting concentration of 1 mg/ml.

variation in specificity (Fig. 3A). Sera from rabbits 13, 14, 15, 17, 18, 19, and 20 reacted with EBs from serovars A, C, I, and J. Serum from rabbit 18 additionally reacted with serovar K EBs, but serum from rabbit 16 reacted only with serovar A EBs. Except for rabbit 19, the intensity of the reaction with the homologous serovar A was relatively constant between rabbits, whereas the intensity of the reaction with heterologous serovars was more varied. In contrast, sera from rabbits immunized with PV1-CtIVA (rabbits 24 to 26), PV1-CtIVB (rabbits 27 to 29), and PV1-CtIVC (rabbits 30 to 32) were more uniformly cross-reactive with B-complex serovars (Fig. 3B). All sera reacted very strongly with serovars D, E, and K. Most sera were immunoreactive with serovars B, Ba, F, and G, though this was usually weaker than the response to serovars D, E, and K and was more variable among the sera. Rabbits 27 and 31 reacted weakly with C-complex serovars (A, C, H, I, and J), and several other rabbits showed evidence of weak reactions with serovar A. In general, reactions were strongest with, but not limited to, B-complex serovars. Sera raised against PV1-XLD did not react with EBs of any serovar (not shown).

The rabbit antisera were tested for the ability to neutralize chlamydial infectivity for HaK cells (14). Antisera to PV1-CtIVA, PV1-CtIVB, and PV1-CtIVC were tested as pools (IVA, IVB, and IVC, respectively), and all showed essentially the same pattern of neutralizing activity (Table 2). All pools neutralized serovars Ba, D, E, and K strongly. Serovar G was neutralized strongly by pools IVB and IVC and to a lesser extent by pool IVA. Serovar A was neutralized weakly by pool IVC but not by pool IVA or IVB. Weak neutralizing activity against serovars B, F, and H was also observed. Interestingly, the sera in pool IVC, from rabbits immunized with PV1-CtIVC, was the most broadly cross-reactive although PV1-CtIVC expresses the shortest VDIV sequence. This finding suggests either that the VDIV sequence is expressed in a more favorable conformation on this hybrid or that a broader cross-reactivity was obtained by focusing the anti-VDIV response to epitopes, possibly immunorecessive, within the highly conserved sequence TTLNPTIAGA.

We also examined the neutralizing activity of antisera raised against the previously described VDI hybrids in more detail. Pooled anti-PV1-Ct7 and anti-PV1-Ct8 sera exhibited high-titer neutralizing activity against the homotypic serovar A (Table 2). In addition, they showed strong neutralizing activity

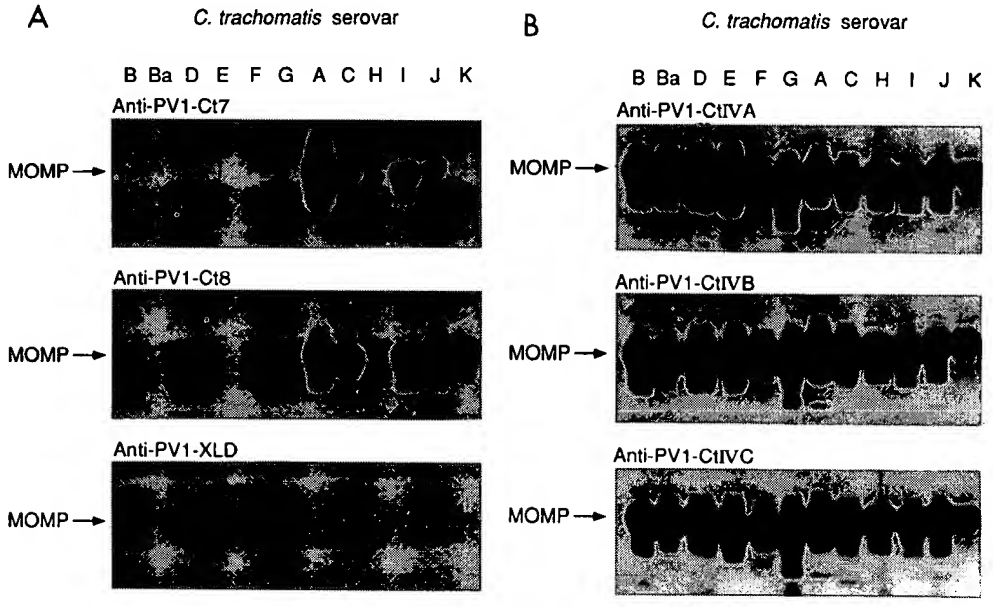


FIG. 2. (A) Specificity of pooled rabbit anti-PV1-Ct7 and anti-PV1-Ct8 antisera for MOMPs from 12 *C. trachomatis* serovars determined by Western blot analysis. Blots made with pooled anti-PV1-XLD antisera are shown as a control. (B) Specificity of pooled rabbit anti-PV1-CtIVA, anti-PV1-CtIVB, and anti-PV1-CtIVC antisera for MOMPs from 12 *C. trachomatis* serovars determined by Western blot analysis. Western blotting was conducted as described previously (14).

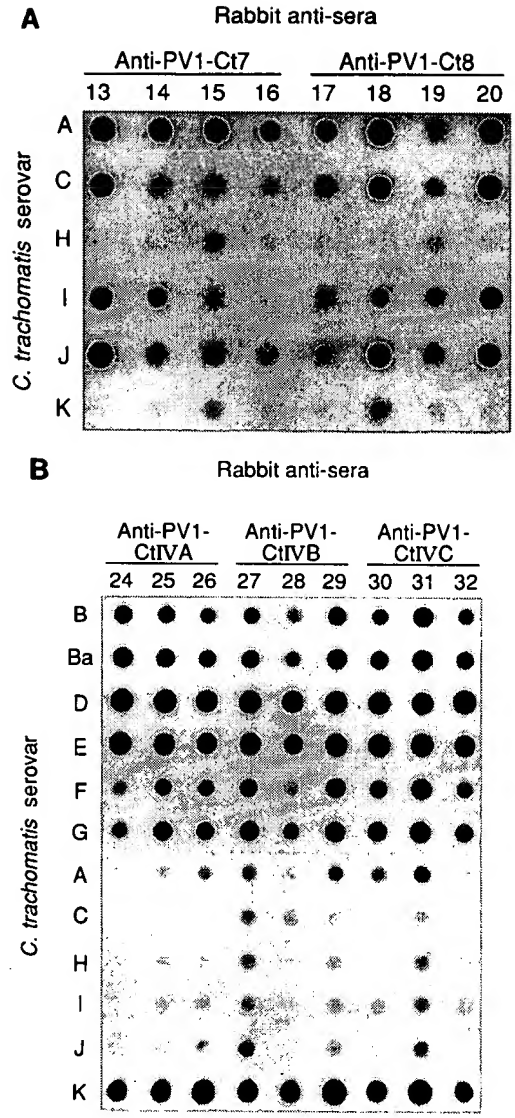


FIG. 3. (A) Specificity by dot immunoblots of rabbit anti-PV1-Ct7 (rabbits 13 to 16) and anti-PV1-Ct8 (rabbits 17 to 20) antisera for EBs of the *C. trachomatis* C-complex serovars (A, C, H, I, and J) and serovar K. (B) Specificity of rabbit anti-PV1-CtIVA (rabbits 24 to 26), anti-PV1-CtIVB (rabbits 27 to 29), and anti-PV1-CtIVC (rabbits 30 to 32) antisera for EBs of 12 *C. trachomatis* serovars by dot immunoblotting. Dot immunoblotting was conducted as described by Zhang et al. (32).

against serovar C, lower activity against serovar I, and weak activity against serovar J.

The immunogenic characteristics of the VDIV hybrids generally conformed to the known properties of the sequence expressed and of MAb DIII-A3, but there were a number of notable exceptions. Anti-VDIV hybrid antisera were species specific in a Western blot against the denatured MOMP, demonstrating that some linear epitopes within the VDIV sequences expressed are conserved species-wide, whereas DIII-A3 recognizes the denatured MOMP of all serovars except K (31). However, these epitopes are clearly masked in the context of the native EB of at least some serovars, as shown by dot immunoblot (in which native MOMP epitopes are retained [32]) or neutralization. The anti-VDIV MAb DIII-A3 recognizes serovars B, Ba, L1, L2, L3, D, E, F, and G by dot immunoblotting (2, 31, 32), whereas anti-VDIV hybrid antisera had relatively weak activity against B, Ba, F, and G but recognized D, E, and K strongly. This was true for all anti-VDIV hybrid antisera, regardless of how well DIII-A3 neutralized the hybrid. Weak activity against serovar A or J in the immunoblot was observed for one or two of the antisera. A similar specificity was evident at the level of neutralization. In particular, serovar K was neutralized strongly, even though it does not retain the VDIV LNPTIAG epitope and is not recognized by DIII-A3. That the antibodies elicited by the VDIV hybrids have a specificity distinct from that of DIII-A3 suggests that the DIII-A3 epitope is relatively immunorecessive in the context of the VDIV hybrids. We interpret the neutralization results to mean that there are stronger neutralization epitopes than those recognized by DIII-A3 within the VDIV sequence and that these epitopes may overlap with but are distinct from LNPTIAG.

It has been reported that synthetic peptides containing the LNPTIAG epitope are also capable of eliciting cross-reactive neutralizing antibodies. Su and Caldwell (24) have described a peptide expressing a VDIV sequence similar to that used here together with a chlamydial T-help epitope. The peptide induced neutralizing antibodies against serovars D (B complex) and G (intermediate complex) but not H (C complex), comparable to the results obtained with the VDIV hybrids; other serovars were not examined. Qu et al. (19) studied the immunogenicity of a peptide expressing the VDI sequence of serovar C and the VDIV sequence of serovar E. The B-complex VDIV sequence expressed on the VDIV hybrids is conserved in serovar E, whereas the serovar A VDI sequence expressed on the VDI hybrids is distinct from the equivalent serovar C sequence. Qu et al. (19) observed that their peptide induced neutralizing antibodies against serovars B, D, E, J, C, L3, and, weakly, F but not against serovars H, I, and A, whereas antisera to the VDI and VDIV hybrids could, in combination, strongly neutralize serovars A, C, I, Ba, D, E, G, and K and weakly neutralize serovars B, F, H, and J. Serovars L1, L2, and L3 were not tested in our study.

Thus, the specificity of the response elicited by the hybrids is distinct from but comparable to the specificity of the response elicited by synthetic peptides. Expressing the VDI and VDIV epitopes on poliovirus hybrids or as synthetic peptides does not substantially change their capacity to induce a cross-reactive immune response. In total, the two classes of hybrids could induce detectable neutralizing responses in rabbits to all 12 serovars tested, indicating that relatively few hybrids would be required for a candidate vaccine to be used against all chlamydial infections. However, the hybrids possess two potentially significant advantages over synthetic peptides. First, the hybrids are powerful immunogens; on a molar basis, the response to 0.5 pmol of hybrid (equivalent to 30 pmol of chlamydial epitope) is similar to that elicited by nanomolar amounts of peptide antigens (14, 19, 24). Second, as discussed previously (14), poliovirus hybrids should be able to induce a much stronger mucosal immune response than a synthetic peptide.

To use such hybrids as a vaccine, it would obviously be necessary to incorporate chlamydial T-cell help epitopes in addition to the B-cell epitopes. Potentially suitable T-cell epitopes are known (e.g., reference 24), and we have previously described expression sites on the poliovirus capsid which can be used to construct a hybrid simultaneously expressing two different epitopes (13, 15). Other sites in the poliovirus capsid proteins may also accommodate the expression of a foreign epitope. In addition, techniques for the expression of entire foreign proteins by poliovirus vectors have recently been described (1, 11), which may make it possible to express mul-

TABLE 2. Neutralization of *C. trachomatis* by rabbit anti-poliovirus-MOMP hybrid antisera[a]

Immunizing antigen	Neutralization titer[b] against *C. trachomatis* serovar:											
	B	Ba	D	E	F	G	A	C	H	I	J	K
PV1-XLD	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	16
PV1-CtIVA	16	256	256	256	<16	64	<16	<16	16	<16	<16	256
PV1-CtIVB	16	256	256	256	16	256	<16	<16	16	<16	<16	1,024
PV1-CtIVC	16	128	256	1,024	16	256	16	<16	16	<16	<16	2,048
PV1-Ct7	ND[c]	ND	ND	ND	ND	ND	1,024	512	<16	64	16	ND
PV1-Ct8	ND	ND	ND	ND	ND	ND	1,024	1,024	<16	64	16	ND

[a] Antisera were tested as pools prepared from all rabbits immunized with each antigen. Four rabbits were used for PV1-Ct7 and PV1-Ct8; three rabbits were used for the other antigens.

[b] Reciprocal of the highest dilution giving at least 50% reduction in chlamydial inclusion-forming units in a neutralization assay conducted with HaK cells as described previously (14).

[c] ND, not done.

tiple B- and T-cell epitopes from a single hybrid. Consequently, the expression by poliovirus vectors of sufficient epitopes to elicit both antichlamydial antibodies and appropriate cognate T-cell help would appear to be technically feasible.

It is clear from the dot blot results that while rabbits respond homogeneously with respect to some chlamydial serovars, the response with respect to several others is variable from rabbit to rabbit. This is particularly evident in the response to the VDI hybrids. Strains of inbred mice have been reported to vary in the response to synthetic peptides expressing the chlamydial epitopes used here, probably because of variations in T-help epitope restriction (19, 23). Nonresponsiveness could be overcome by the use of keyhole limpet hemocyanin as a carrier or the provision of a chlamydial T-help epitope. The rabbit-to-rabbit variation observed in this study is probably not due to lack of effective T-cell help, since this would be provided by the poliovirus vector. Indeed, all rabbits mounted a strong antipoliovirus response (data not shown). Rather, the variation is probably due to differences in the rabbit B-cell antigen receptor repertoire and ability to recognize chlamydial B-cell epitopes. We have observed that in rabbits immunized with the VDI hybrids, cross-reactive antisera preferentially recognize epitopes in the chlamydial sequence TTSDVAGLEK, whereas antisera with greater specificity for serovar A preferentially recognize epitopes in the chlamydial sequence VAGLEKD PVA (12a). It may be possible to elicit a more consistently cross-reactive response by modifying the expressed VDI sequence so that responses are preferentially directed toward the sequence TTSDVAGLEK, for instance by changing or deleting the VDI amino acids PVA. This is supported by the observation that antisera to PV1-Ct8, which expresses TTSD VAGLEKDP, tend to be more cross-reactive than antisera to PV1-Ct7, which expresses TTSDVAGLEKDPVA.

We have previously proposed that the use of poliovirus-chlamydia hybrids will allow us to address the role of mucosal immunity in chlamydial infections and have shown that VDI hybrids were viable and immunogenic. We have shown here that these hybrids can elicit neutralizing antibodies against several C-complex serovars (A, C, I, and J). It is also possible to express VDIV sequences on poliovirus hybrids that are viable and immunogenic. The VDIV hybrids elicit a broadly cross-reactive immune response which is able to neutralize many B-complex serovars. Thus, by using only two hybrids, it is possible to elicit neutralizing antibodies against most chlamydial serovars. This result, combined with the ability of poliovirus to induce a mucosal immune response, should greatly facilitate the study of the role of mucosal immunity in both trachoma and chlamydial STDs.

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REFERENCES

1. **Andino, R., D. Silvera, S. D. Suggett, P. L. Achacoso, C. J. Miller, D. Baltimore, and M. B. Feinberg.** 1994. Engineering poliovirus as a vaccine vector for the expression of diverse antigens. Science **265**:1448–1451.
2. **Baehr, W., Y.-X. Zhang, T. Joseph, H. Su, F. E. Nano, K. D. E. Everett, and H. D. Caldwell.** 1988. Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. Proc. Natl. Acad. Sci. USA **85**:4000–4004.
3. **Caldwell, H. D., J. Kromhout, and J. Schachter.** 1981. Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. Infect. Immun. **31**:1161–1176.
4. **Conlan, J. W., I. N. Clarke, and M. E. Ward.** 1988. Epitope mapping with solid-phase peptides: identification of type-, subspecies-, species-, and genus-reactive antibody binding domains on the major outer membrane protein of *Chlamydia trachomatis*. Mol. Microbiol. **2**:673–679.
5. **Dawson, C. R., B. R. Jones, and M. L. Tarizzo.** 1981. Guide to trachoma control and prevention of blindness, p. 38–47. World Health Organization, Geneva.
6. **Golding, S. M., R. S. Hedger, P. Talbot, and J. Watson.** 1976. Radial immunodiffusion and serum neutralisation techniques for the assay of antibodies to SVD. Res. Vet. Sci. **20**:142–147.
7. **Grayston, J. T.** 1971. Trachoma vaccine, p. 311–315 *In* D. Washington (ed.), International Conference on the Application of Vaccines Against Viral, Rickettsial and Bacterial Diseases of Man. Pan American Health Organization, Washington, D.C.
8. **Grayston, J. T., and S.-P. Wang.** 1975. New knowledge of chlamydiae and the diseases they cause. J. Infect. Dis. **132**:87–104.
9. **Grayston, J. T., S.-P. Wang, L.-J. Yeh, and C.-C. Kuo.** 1985. Importance of reinfection in the pathogenesis of trachoma. Rev. Infect. Dis. **7**:717–725.
10. **Lucero, M. E., and C.-C. Kuo.** 1985. Neutralization of *Chlamydia trachomatis* cell culture infection by serovar specific monoclonal antibodies. Infect. Immun. **50**:595–597.
11. **Mattion, N. M., P. A. Reilly, S. J. DiMichelle, J. C. Crowley, and C. Weeks-Levy.** 1994. Attenuated poliovirus strain as a live vector: expression of regions of the rotavirus outer capsid protein VP7 by using recombinant Sabin 3 viruses. J. Virol. **68**:3925–3933.
12. **Morrison, R. P., D. S. Manning, and H. D. Caldwell.** 1992. Immunology of *Chlamydia trachomatis* infections: immunoprotective and immunopathogenetic responses, p. 57–84. *In* T. C. Quinn (ed.), Sexually transmitted diseases. Raven Press Ltd., New York.

12a. **Murdin, A. D., et al.** Unpublished data.

13. **Murdin, A. D., H.-H. Lu, M. G. Murray, and E. Wimmer.** 1992. Poliovirus antigenic hybrids simultaneously expressing antigenic determinants from all three serotypes. J. Gen. Virol. **73**:607–611.
14. **Murdin, A. D., H. Su, D. S. Manning, M. H. Klein, M. J. Parnell, and H. D. Caldwell.** 1993. A poliovirus hybrid expressing a neutralization epitope from the major outer membrane protein of *Chlamydia trachomatis* is highly immunogenic. Infect. Immun. **61**:4406–4414.
15. **Murdin, A. D., and E. Wimmer.** 1989. Construction of a poliovirus type 1/type 2 antigenic hybrid by manipulation of neutralization antigenic site II. J. Virol. **63**:5251–5257.
16. **Murray, M. G., M. Arita, N. Kawamura, A. Nomoto, and E. Wimmer.** 1988. Poliovirus type 1/type 3 antigenic hybrid virus constructed *in vitro* elicits type 1 and type 3 neutralizing antibodies in rabbits and monkeys. Proc. Natl.

Acad. Sci. USA 85:3203–3207.
17. **Ogra, P. L., and S. S. Ogra.** 1973. Local antibody response to poliovaccine in the human female genital tract. J. Immunol. **110**:1307–1311.
18. **Peeling, R., I. W. McClean, and R. C. Brunham.** 1984. In vitro neutralization of *Chlamydia trachomatis* with monoclonal antibody to an epitope on the major outer membrane protein. Infect. Immun. **46**:484–488.
19. **Qu, Z., X. Cheng, L. M. de la Maza, and E. M. Peterson.** 1994. Analysis of the humoral response elicited in mice by a chimeric peptide representing variable segments I and IV of the major outer membrane protein of *Chlamydia trachomatis*. Vaccine **12**:557–564.
20. **Schachter, J.** 1978. Chlamydial infections. N. Engl. J. Med. **298**:540–548.
21. **Schachter, J.** 1988. The intracellular life of Chlamydia. Curr. Top. Microbiol. Immunol. **138**:109–139.
22. **Stephens, R. S., E. A. Wagar, and G. K. Schoolnik.** 1988. High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of *Chlamydia trachomatis*. J. Exp. Med. **167**: 817–831.
23. **Su, H., and H. D. Caldwell.** 1992. Immunogenicity of a chimeric peptide corresponding to T-helper and B-cell epitopes of the *Chlamydia trachomatis* major outer membrane protein. J. Exp. Med. **175**:227–235.
24. **Su, H., and H. D. Caldwell.** 1993. Immunogenicity of a synthetic oligopeptide corresponding to antigenically common T-helper and B-cell neutralizing epitopes of the major outer membrane protein of *Chlamydia trachomatis*. Vaccine **11**:1159–1166.
25. **Taylor, H. R., S. L. Johnson, R. A. Prendergast, J. Schachter, C. R. Dawson, and A. M. Silverstein.** 1982. An animal model of trachoma. II. The importance of repeated infection. Invest. Ophthalmol. Visual Sci. **23**:507–515.
26. **Taylor, H. R., S. L. Johnson, J. Schachter, H. D. Caldwell, and R. A. Prendergast.** 1987. Pathogenesis of trachoma: the stimulus for inflammation. J. Immunol. **138**:3023–3027.
27. **van der Werf, S., J. Bradley, E. Wimmer, F. W. Studier, and J. J. Dunn.** 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83**:2330–2334.
28. **Washington, A. E., R. E. Johnson, and L. L. Sanders, Jr.** 1987. *Chlamydia trachomatis* infections in the United States: what are they costing us? JAMA **257**:2070–2072.
29. **Washington, A. E., and P. Katz.** 1991. Cost of and payment source for pelvic inflammatory disease. JAMA **266**:2565–2569.
30. **Yuan, Y., Y.-X. Zhang, N. G. Watkins, and H. D. Caldwell.** 1989. Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. Infect. Immun. **57**:1040–1049.
31. **Zhang, Y.-X., S. J. Stewart, and H. D. Caldwell.** 1989. Protective monoclonal antibodies to *Chlamydia trachomatis* serovar- and serogroup-specific major outer membrane protein determinants. Infect. Immun. **57**:636–638.
32. **Zhang, Y.-X., S. Stewart, T. Joseph, H. R. Taylor, and H. D. Caldwell.** 1987. Protective monoclonal antibodies recognise epitopes located on the major outer membrane protein of *Chlamydia trachomatis*. J. Immunol. **138**:575–581.

CLINICAL PRACTICE

Genital Chlamydial Infections

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This Journal feature begins with a case vignette highlighting a common clinical problem. Evidence supporting various strategies is then presented, followed by a review of formal guidelines, when they exist. The article ends with the author's clinical recommendations.

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A 19-year-old woman visits her primary care provider for counseling about contraception. She became sexually active one year previously and has had a new sexual partner for the past three months. Her partner currently uses a condom intermittently for contraception, and she inquires about oral contraceptives. She reports no medical problems and is in good health. Her physical examination is unremarkable. Is testing for *Chlamydia trachomatis* indicated?

THE CLINICAL PROBLEM

Chlamydia trachomatis is the most common bacterial cause of sexually transmitted infections in the United States, responsible for an estimated 3 million new infections each year.[1,2] The cost of care for untreated chlamydial infections and their complications is estimated to exceed $2 billion annually.[3]

CLINICAL PRESENTATION

As many as 85 to 90 percent of *C. trachomatis* infections in men and women are asymptomatic.[4,5] Asymptomatic infections can persist for several months.[5] Despite the frequent absence of symptoms, at least one third of women have local signs of infection on examination.[5] The two most commonly reported signs are mucopurulent discharge from the cervix and hypertrophic cervical ectopy (Fig. 1). Signs and symptoms in men include urethral discharge of mucopurulent or purulent material, dysuria, or urethral pruritus.

Clinical manifestations of *C. trachomatis* infections in women include acute urethral syndrome, urethritis, bartholinitis, cervicitis, upper genital tract infection (endometritis, salpingo-oophoritis, or pelvic inflammatory disease), perihepatitis (Fitz-Hugh–Curtis syndrome), and reactive arthritis.[5] Symptoms depend on the site of infection. Infection of the urethra and lower genital tract may cause dysuria, abnormal vaginal discharge, or postcoital bleeding, whereas infection of the upper genital tract (e.g., endometritis or salpingitis) may be manifested as irregular uterine bleeding and abdominal or pelvic discomfort.

In women, untreated chlamydial infection can lead to severe reproductive complications. *C. trachomatis* is an important causal agent in pelvic inflammatory disease, with sequelae including infertility, ectopic pregnancy, and chronic pelvic pain.[6] Up to two thirds of cases of tubal-factor infertility and one third of cases of ectopic pregnancy may be attributable to *C. trachomatis* infection.[7] Chlamydial infection during pregnancy is associated with a number of adverse outcomes of pregnancy including preterm labor, premature rupture of the membranes, low birth weight, neonatal death, and postpartum endometritis.[8,9]

Chlamydial infection during pregnancy may be transmitted to the infant during de-

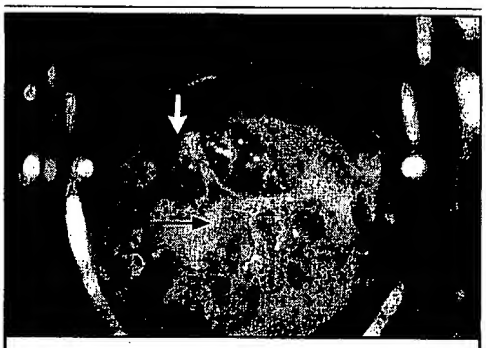

Figure 1. Cervical Ectropion (White Arrow) with Mucopurulent Cervicitis (Black Arrow).
Photograph courtesy of Dr. Marc Steben.

livery.[10] An infant born to a mother with active infection has a risk of acquiring infection at any anatomical site of 50 to 75 percent. Approximately 30 to 50 percent of infants born to chlamydia-positive mothers will have conjunctivitis, and at least 50 percent of infants with chlamydial conjunctivitis will also have nasopharyngeal infection. Chlamydial pneumonia develops in about 30 percent of infants with nasopharyngeal infection.[5]

In men, the most common clinical manifestation of *C. trachomatis* infection is nongonococcal urethritis. In fact, *C. trachomatis* causes approximately 35 to 50 percent of all cases of nongonococcal urethritis in heterosexual men. Symptoms of nongonococcal urethritis may develop after an incubation period of 7 to 21 days and include dysuria and mild-to-moderate whitish or clear urethral discharge. In most cases, physical examination reveals no abnormalities other than the discharge. Other clinical syndromes in men include acute epididymitis, acute proctitis, acute proctocolitis, conjunctivitis, and Reiter's syndrome.[5] Male infertility, chronic prostatitis, and urethral strictures are possible results of infection. Both Reiter's syndrome (urethritis, conjunctivitis, arthritis, and mucocutaneous lesions) and reactive tenosynovitis or arthritis (without the other components of Reiter's syndrome) have been associated with genital *C. trachomatis* infection.[5] Infection with *C. trachomatis* is also believed to be a cofactor for the transmission of human immunodeficiency virus in both men and women.[11]

EPIDEMIOLOGY OF CHLAMYDIAL INFECTIONS

The prevalence of chlamydia depends on the characteristics of the population studied. Reported prevalence rates in the United States have ranged from 2 to 7 percent among female college students and 4 to 12 percent among women attending a family planning clinic to 6 to 20 percent among men and women attending a clinic for sexually transmitted diseases or persons entering correctional facilities.[5,12] In the United Kingdom, recent data suggest that the rate of infection among young women exceeds 10 percent.[13] The prevalence of *C. trachomatis* infection is highest in groups of persons who are the least likely to see a clinician. Prevalence rates have declined in geographic areas where screening programs have been implemented.[14]

Risk factors for chlamydial infection in sexually active women include a young age (less than 25 years and, in particular, less than 20 years), intercourse at an early age, having more than one sexual partner, involvement with a new sexual partner, being unmarried, black race, a history of or coexistent sexually transmitted infection, cervical ectopy, and inconsistent use of barrier contraceptive methods.[15,16] Young age is the factor that is most strongly associated with infection (relative risk among women younger than 25 years as compared with older women, 2.0 to 3.5).[17] This association is largely attributable to the higher level of sexual activity among younger women. Also, in younger women, the squamocolumnar junction of the cervix often lies well out on the ectocervix, forming a bright red central zone of ectopic columnar epithelium called an ectropion (Fig. 1); this ectopy provides a larger target area for chlamydial infection than is present in older women.[18]

STRATEGIES AND EVIDENCE

SCREENING

Screening in Women

There is good evidence that screening women who are at risk for *C. trachomatis* infection can prevent reproductive sequelae by reducing the rate of pelvic inflammatory disease.[15] The strongest evidence supporting screening in women comes from a large randomized trial of screening and treatment at a health maintenance organization in Seattle.[19] Participants were unmarried, asymptomatic women (18 to 34 years of age) who were considered to have a high risk of *C. trachomatis* infection on the basis of a scoring system that included as risk factors a young age (less than 25 years), black race, nulligravidity, douching, and two or more sexual partners during the previous 12 months. By the end of the

follow-up period, there were 9 verified cases of pelvic inflammatory disease in the screened group (8 per 10,000 woman-months of follow-up) and 33 cases in the usual-care group (18 per 10,000 woman-months; relative risk in the screened group, 0.44; 95 percent confidence interval, 0.20 to 0.90). Long-term adverse outcomes of chlamydial infection were not addressed in this study.

In addition, two ecologic studies (studies evaluating associations between types of exposure and outcomes in populations rather than individual persons), conducted in Sweden, showed that the rates of both ectopic pregnancies and pelvic inflammatory disease were reduced in communities after screening for chlamydial infection was adopted.[20,21] However, it is possible that the lower prevalence of adverse outcomes in these studies was due to factors other than screening, such as increased use of barrier contraceptives and reductions in risk-taking behavior.

Although data from randomized trials of screening for chlamydial infection during pregnancy are lacking, there is some evidence that screening high-risk women for *C. trachomatis* during pregnancy can reduce the rate of adverse outcomes of pregnancy. Two observational studies showed associations between the treatment of chlamydial infections during pregnancy and improved outcomes of pregnancy, including lower rates of premature rupture of the membranes, low birth weight, births of infants who were small for their gestational age, and neonatal death.[22,23]

Screening in Men

The U.S. Preventive Services Task Force found no direct evidence to determine whether screening asymptomatic men is effective for reducing the incidence of new infections in women, and it could not determine the balance of the harms and benefits of screening men.[15]

Testing Methods

The gold standard for the diagnosis of *C. trachomatis* infection was traditionally a culture of a swab from the endocervix in women or the urethra in men. However, the methodologic challenges of culturing this organism led to the development of non–culture-based tests. Whereas early non–culture-based tests, including antigen-detection tests and nonamplified nucleic acid hybridization, were limited by their failure to detect a substantial proportion of infections,[24] newer tests that amplify and detect *C. trachomatis*–specific DNA or RNA sequences[25] (including polymerase chain reaction, ligase chain reaction, and transcription-mediated amplification of RNA) are substantially more sensitive than the first-generation non–culture-based tests (80 to 91 percent, depending on the site from which the specimen is obtained, vs. 62 to 75 percent), when culture is used as the gold standard.[25] The sensitivity is slightly lower when these newer tests are performed on urine specimens rather than endocervical specimens, but the specificity is high for all types of specimens (range, 94 to nearly 100 percent).[25] The majority of nucleic acid–amplification tests have been approved by the Food and Drug Administration for the detection of *C. trachomatis* (and *Neisseria gonorrhoeae*) in urine from both men and women, providing a noninvasive testing method.[25] Limitations of nucleic acid–amplification tests include their relatively high cost and the requirement for a suitable laboratory.[25]

The most recent addition to the testing armamentarium is the use of specimens collected by patients.[26,27] Amplification testing of vaginal or urethral swab specimens collected by patients has a sensitivity and specificity similar to those of amplification testing of specimens collected by clinicians, and studies indicate that patients prefer this method to the standard collection methods.[28,29]

TREATMENT

The treatment of chlamydial infection depends on the clinical syndrome (Table 1).[2] Effective and low-cost treatments for genital chlamydial infection are available for the most common clinical syndromes (nongonococcal urethritis in men and mucopurulent cervicitis in women). In a randomized trial, the efficacy of a seven-day course of doxycycline was equivalent to that of a single dose of azithromycin; both resulted in cure rates of more than 95 percent among men and nonpregnant women.[30] Sexual partners should be notified, examined, and treated for chlamydia and any other identified or suspected sexually transmitted disease. Patients and their partners should be instructed to refrain from sexual intercourse until therapy is completed (specifically, until seven days after a single-dose regimen or until the completion of a seven-day regimen).[2]

Infection during Pregnancy

A Cochrane review of 11 randomized trials for the treatment of chlamydia during pregnancy concluded that amoxicillin was as effective as oral erythro-

Table 1. Common Clinical Syndromes and Their Treatment.

Syndrome	Recommended Regimens
Men	
Nongonococcal urethritis	Azithromycin, 1 g orally (single dose), or doxycycline, 100 mg orally 2 times a day for 7 days
Recurrent or persistent urethritis	Metronidazole, 2 g orally (single dose), plus erythromycin base, 500 mg orally 4 times a day for 7 days, or erythromycin ethylsuccinate, 800 mg orally 4 times a day for 7 days
Epididymitis	Ceftriaxone, 250 mg intramuscularly (single dose), plus doxycycline, 100 mg orally 2 times a day for 10 days
Women	
Mucopurulent cervicitis	Azithromycin, 1 g orally (single dose), or doxycycline, 100 mg orally 2 times a day for 7 days
Chlamydia during pregnancy	Erythromycin base, 500 mg orally 4 times a day for 7 days, or amoxicillin, 500 mg orally 3 times a day for 7 days, or azithromycin, 1 g orally (single dose)
Pelvic inflammatory disease	
Outpatient	Ofloxacin, 400 mg orally 2 times a day for 14 days, or levofloxacin, 500 mg orally once a day for 14 days, with or without metronidazole, 500 mg orally 2 times a day for 14 days; otherwise, ceftriaxone, 250 mg intramuscularly (single dose), or cefoxitin, 2 g intramuscularly (single dose), plus probenecid, 1 g orally, plus doxycycline, 100 mg orally 2 times a day for 14 days, with or without metronidazole, 500 mg orally 2 times a day for 14 days
Inpatient*	Cefotetan, 2 g intravenously every 12 hours, or cefoxitin, 2 g intravenously every 6 hours, plus doxycycline, 100 mg orally or intravenously every 12 hours; otherwise, clindamycin, 900 mg intravenously every 8 hours, plus gentamicin, 2 mg per kg of body weight loading dose intravenously, then 1.5 mg per kg every 8 hours; daily administration of a single dose may be substituted

* Therapy for pelvic inflammatory disease should be continued for 24 to 48 hours after clinical improvement occurs and should consist of continuous oral therapy with doxycycline, 100 mg orally twice a day, or clindamycin, 450 mg orally 4 times a day, for a total of 14 days.

mycin.[31] Several small trials comparing oral azithromycin with these therapies have shown similar cure rates and acceptability for azithromycin.[32,33]

Pelvic Inflammatory Disease

Although pelvic inflammatory disease is thought to be a polymicrobial infection, *C. trachomatis* is one of the more common pathogens involved. The minimal criteria for a diagnosis of pelvic inflammatory disease include uterine–adnexal tenderness or cervical-motion tenderness.[2] Some studies suggest that atypical presentations of pelvic inflammatory disease, including discomfort without appreciable tenderness, abnormal uterine bleeding, and abnormal vaginal discharge, are often associated with infection and inflammation in the upper genital tract (i.e., endometritis and salpingitis).[34,35] Chlamydial pelvic inflammatory disease tends to have a more insidious onset than pelvic inflammatory disease caused by *N. gonorrhoeae* or other more virulent organisms. However, the damage to the fallopian tube can be as great or greater with chlamydia, especially with repeated infections.[36]

Because of the risks of infertility and other sequelae of pelvic inflammatory disease, clinicians should have a low threshold for the prompt institution of treatment in women who are at risk for chlamydial infection. The delay of antibiotic therapy is associated with an increased risk of adverse outcomes.[37] The PID [Pelvic Inflammatory Disease] Evaluation and Clinical Health (PEACH) study, a randomized trial comparing inpatient therapy consisting of cefoxitin and doxycycline with similar outpatient therapy, demonstrated that outpatient therapy for uncomplicated pelvic inflammatory disease (without tubo-ovarian abscess or severe illness) was as effective as intravenous inpatient therapy in terms of fertility and other long-term health outcomes, including the prevention of ectopic pregnancy and chronic pelvic pain.[38]

AREAS OF UNCERTAINTY

There continues to be uncertainty regarding whom to screen and how frequently to do so. There is little evidence of the effectiveness of screening in asymptomatic women who are not in high-risk groups.[15] Screening on the basis of age (less than 25 years) appears to be effective even in areas where the prevalence of chlamydial infection is low to moderate

(3 to 6 percent). In a longitudinal cohort study of screening in 3202 high-risk, inner-city young women, chlamydial infection was detected in 24.1 percent; the median time to new infection was slightly more than 7 months, and the median time to a repeated positive test was 6.3 months. On the basis of these results, it was recommended that all young, sexually active women be screened every six months.[39] It is unclear, however, whether these findings can be generalized to populations with a lower prevalence of infection.

Level I evidence (from randomized trials) is also lacking regarding the effectiveness of the screening and treatment of pregnant women in populations with a low prevalence of chlamydial infection. In addition, the balance of benefits and harms (including false-positive test results and the inappropriate use of antibiotics) has not been assessed.[15]

Given the high prevalence of asymptomatic infections in the population, some experts advocate for the routine screening of young men as the next important step toward reduced rates of infections and complications.[40,41] Although there is strong evidence that treatment can eradicate *C. trachomatis* infection in men, there are no studies demonstrating that the screening of asymptomatic men can reduce the rates of acute infection and adverse outcomes in men or in women. Cost-effectiveness analyses have suggested that there is an economic benefit to society of screening for *C. trachomatis*, as compared with not screening, in high-risk women.[42,43] However, the cost effectiveness of the screening of men and low-risk women is debatable and will depend on the prevalence of infections, the ease and cost of specimen collection, the cost of testing, the characteristics of the diagnostic tests (e.g., their sensitivity and specificity), and the short- and long-term adverse outcomes that are prevented. Additional research is needed to determine the optimal interval between screenings and to compare the universal screening of all sexually active women younger than 25 years of age with screening based on the presence of additional risk factors in populations with a range of prevalence rates.

It remains uncertain whether the routine use of urine specimens or specimens collected by patients would improve compliance with testing and treatment. Also, it is unclear whether the empirical treatment of the sexual partners of patients with chlamydial infections is preferable to the screening of these partners. Some experts suggest that providing patients with prescriptions for empirical treatment to deliver to their sexual partners will reduce the rate of reinfection,[44] but this hypothesis remains unproven. In a recent trial in which patients were randomly assigned to either patient-delivered treatment for partners (patients were asked to deliver a dose of azithromycin to each of their sexual partners) or self-referral (patients were asked to refer their sexual partners for treatment), the risk of reinfection was nonsignificantly lower in the group assigned to patient-delivered treatment (odds ratio for reinfection, 0.8; 95 percent confidence interval, 0.6 to 1.1).[45]

GUIDELINES

Guidelines from several professional societies, the Centers for Disease Control and Prevention, and the U.S. Preventive Services Task Force are summarized in Table 2.[46-50] All groups suggest that clinicians screen routinely for *C. trachomatis* in all sexually active women less than 25 years of age and in other asymptomatic women who are at increased risk for infection.

CONCLUSIONS AND RECOMMENDATIONS

Screening for *C. trachomatis* infection is indicated in sexually active women with risk factors for this infection, including an age of less than 25 years, inconsistent use of barrier contraceptives, a new sexual partner, more than one sexual partner, cervical ectopy, and a history of or coexisting sexually transmitted disease. The patient described in the vignette has some of these risk factors. Electing not to screen her would place her at risk for adverse outcomes, including ascending infection (pelvic inflammatory disease) and infertility, chronic pelvic pain, and ectopic pregnancy. Annual screening is reasonable, although more frequent testing may be indicated in areas of high prevalence or in women with several risk factors. Information on prevalence (the rates of positive tests) can often be obtained from microbiology laboratories. The use of barrier contraception (e.g., condoms) as a method of prevention should be discussed with all patients. If a screening test is positive for *C. trachomatis*, I would treat the patient with doxycycline or azithromycin. Retesting (a "test of cure") after treatment with recommended regimens is not indicated unless compliance is in question, symptoms are present, or reinfection is suspected.[2] Rescreening for chlamydia is recom-

Table 2. Guidelines from Professional Societies and Federal Agencies.

Organization	Who Should Be Screened	Timing or Frequency of Screening
American Academy of Pediatrics[46]	All young, sexually active women	Perform annually
American College of Obstetricians and Gynecologists[47]	All young sexually active women and other women at high risk for infection	Perform routinely (interval not specified)
American College of Preventive Medicine[48]	All sexually active women with risk factors (age ≤25 yr, new male sexual partner or two or more sexual partners in previous year, inconsistent use of barrier contraceptives, history of sexually transmitted disease, black race, cervical ectopy)	Perform annually
	Pregnant women	Perform during first trimester in all women, third trimester if risk factors present
American Medical Association[49]	All young, sexually active women	Perform annually
Canadian Task Force on Preventive Health Care[50]	Persons in high-risk groups (sexually active women <25 yr of age, men and women with a new sexual partner or more than one partner in the previous year, women who use nonbarrier contraceptive methods)	Not specified
	Pregnant women	Perform during first trimester in all women
Centers for Disease Control and Prevention[2]*	All sexually active women <20 yr of age; women 20–24 yr of age if one of the following risk factors is present: inconsistent use of barrier contraceptives or a new sexual partner or multiple sexual partners during the previous 3 mo; women >24 yr of age if both risk factors are present	Perform annually
	Pregnant women	Perform during first prenatal visit in all women, third trimester if high risk (<25 yr of age or other risk factors)
U.S. Preventive Services Task Force[15]†	All sexually active women ≤25 yr of age and other asymptomatic women at increased risk for infection	Perform routinely, optimal interval uncertain
	All asymptomatic pregnant women ≤25 yr of age and others at increased risk for infection	Perform routinely, optimal interval uncertain

* The guidelines of the Centers for Disease Control and Prevention are available at http://www.cdc.gov/std/treatment/rr5106.pdf.
† The guidelines of the U.S. Preventive Services Task Force are available at http://www.ahrq.gov/clinic/uspstf/uspschlm.htm. The task force recommends routine screening in the groups listed and notes that there is insufficient evidence to make a recommendation for or against screening in asymptomatic men.

mended when patients present for care within 12 months after a positive test.[2]

The timely treatment of the patient's sexual partners is also essential in order to reduce the risk of reinfection. The sexual partners should be evaluated, tested, and treated if they have had sexual contact with the patient during the 60 days preceding the diagnosis. Treatment for sexual partners that is delivered by the patient for the prevention of repeated infection has efficacy similar to that of self-referral and is a reasonable approach.

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REFERENCES

1. Sexually transmitted disease surveillance, 2001. Atlanta: Centers for Disease Control and Prevention, September 2002.
2. Sexually transmitted diseases treatment guidelines 2002. MMWR Recomm Rep 2002; 51(RR-6):30-6.
3. Eng Tr, Butler WT, eds. The hidden epidemic: confronting sexually transmitted diseases. Washington, D.C.: National Academy Press, 1997.
4. Cecil JA, Howell MR, Tawes JJ, et al. Features of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection in male army recruits. J Infect Dis 2001;184:1216-9.
5. Stamm WE. *Chlamydia trachomatis* infections of the adult. In: Holmes KK, Mårdh P-A, Sparling PF, et al., eds. Sexually transmitted diseases. 3rd ed. New York: McGraw-Hill, 1999:407-22.
6. Westrom L, Joesoef R, Reynolds G, Hagdu A, Thompson SE. Pelvic inflammatory disease and fertility: a cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. Sex Transm Dis 1992; 19:185-92.
7. Paavonen J, Eggert-Kruse W. Chlamydia trachomatis: impact on human reproduction. Hum Reprod Update 1999;5:433-47.
8. Mardh PA. Influence of infection with

Chlamydia trachomatis on pregnancy outcome, infant health and life-long sequelae in infected offspring. Best Pract Res Clin Obstet Gynaecol 2002;16:847-64.
9. Andrews WW, Goldenberg RL, Mercer B, et al. The Preterm Prediction Study: association of second-trimester genitourinary chlamydia infection with subsequent spontaneous preterm birth. Am J Obstet Gynecol 2000;183:662-8.
10. Jain S. Perinatally acquired Chlamydia trachomatis associated morbidity in young infants. J Matern Fetal Med 1999;8:130-3.
11. Fleming DT, Wasserheit JN. From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. Sex Transm Infect 1999;75:3-17.
12. Hardick J, Hsieh Y, Tulloch S, Kus J, Tawes J, Gaydos CA. Surveillance of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in women in detention in Baltimore, Maryland. Sex Transm Dis 2003;30:64-70.
13. Tobin JM. Chlamydia screening in primary care: is it useful, affordable and universal? Curr Opin Infect Dis 2002;15:31-6.
14. Herrmann B, Egger M. Genital Chlamydia trachomatis infections in Uppsala County, Sweden, 1985-1993: declining rates for how much longer? Sex Transm Dis 1995;22: 253-60.
15. U.S. Preventive Services Task Force. Screening for chlamydial infection: recommendations and rationale. Am J Prev Med 2001;20:Suppl:90-4.
16. Gaydos CA, Howell R, Pare B, et al. *Chlamydia trachomatis* infections in female military recruits. N Engl J Med 1998;339:739-44.
17. Gaydos CA, Howell MR, Quinn TC, McKee KT Jr, Gaydos JC. Sustained high prevalence of *Chlamydia trachomatis* infections in female Army recruits. Sex Transm Dis 2003; 30:539-44.
18. Faro S, Soper DE, eds. Infectious diseases in women. Philadelphia: W.B. Saunders, 2001:261-2.
19. Scholes D, Stergachis A, Heidrich FE, Andrilla H, Holmes KK, Stamm WE. Prevention of pelvic inflammatory disease by screening for cervical chlamydial infection. N Engl J Med 1996;334:1362-6.
20. Kamwendo F, Forslin L, Bodin L, Danielsson D. Decreasing incidences of gonorrhea- and chlamydia-associated acute pelvic inflammatory disease: a 25-year study from an urban area of central Sweden. Sex Transm Dis 1996;23:384-91.
21. Egger M, Low N, Smith GD, Lindblom B, Herrmann B. Screening for chlamydial infections and the risk of ectopic pregnancy in a county in Sweden: ecological analysis. BMJ 1998;316:1776-80.
22. Ryan GM Jr, Abdella TN, McNeeley SG, Baselski VS, Drummond DE. *Chlamydia trachomatis* infection in pregnancy and effect of treatment on outcome. Am J Obstet Gynecol 1990;162:34-9.
23. Cohen I, Veille J-C, Calkins BM. Improved pregnancy outcomes following successful treatment of chlamydial infection. JAMA 1990;263:3160-3.
24. Guaschino S, De Seta F. Update on *Chlamydia trachomatis*. Ann N Y Acad Sci 2000; 900:293-300.
25. Johnson RE, Newhall WJ, Papp JR, et al. Screening tests to detect *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections — 2002. MMWR Recomm Rep 2002;51(RR-15): 1-38.
26. Wiesenfeld HC, Lowry DL, Heine RP, et al. Self-collection of vaginal swabs for the detection of Chlamydia, gonorrhea, and trichomoniasis: opportunity to encourage sexually transmitted disease testing among adolescents. Sex Transm Dis 2001;28:321-5.
27. Gaydos CA, Rompalo AM. The use of urine and self-obtained vaginal swabs for the diagnosis of sexually transmitted diseases. Curr Infect Dis Rep 2002;4:148-57.
28. Verhoeven V, Avonts D, Van Royen P, Denekens J. Self-collection of vaginal swab specimens: the patient's perception. Sex Transm Dis 2002;29:426.
29. Newman SM, Nelson MB, Gaydos CA, Friedman HB. Female prisoners' preferences of collection methods for testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection. Sex Transm Dis 2003;30:306-9.
30. Martin DH, Mroczkowski TF, Dalu ZA, et al. A controlled trial of a single dose of azithromycin for the treatment of chlamydial urethritis and cervicitis. N Engl J Med 1992; 327:921-5.
31. Brocklehurst P, Rooney G. Interventions for treating genital chlamydia trachomatis infection in pregnancy. Cochrane Database Syst Rev 2000;2:CD000054.
32. Jacobson GF, Autry AM, Kirby RS, Liverman EM, Motley RU. A randomized controlled trial comparing amoxicillin and azithromycin for the treatment of Chlamydia trachomatis in pregnancy. Am J Obstet Gynecol 2001;184:1352-4.
33. Kacmar J, Cheh E, Montagno A, Peipert JF. A randomized trial of azithromycin versus amoxicillin for the treatment of Chlamydia trachomatis in pregnancy. Infect Dis Obstet Gynecol 2001;9:197-202.
34. Wiesenfeld HC, Hillier SL, Krohn MA, et al. Lower genital tract infection and endometritis: insight into subclinical pelvic inflammatory disease. Obstet Gynecol 2002; 100:456-63.
35. Korn AP, Bolan G, Padian N, Ohn-Smith M, Schachter J, Landers DV. Plasma cell endometritis in women with symptomatic bacterial vaginosis. Obstet Gynecol 1995;85:387-90.
36. Hillis SD, Owens LM, Marchbanks PA, Amsterdam LF, Mac Kenzie WR. Recurrent chlamydial infections increase the risks of hospitalization for ectopic pregnancy and pelvic inflammatory disease. Am J Obstet Gynecol 1997;176:103-7.
37. Hillis SD, Joesoef R, Marchbanks PA, Wasserheit JN, Cates W Jr, Westrom L. Delayed care of pelvic inflammatory disease as a risk factor for impaired fertility. Am J Obstet Gynecol 1993;168:1503-9.
38. Ness RB, Soper DE, Holley RL, et al. Effectiveness of inpatient and outpatient treatment strategies for women with pelvic inflammatory disease: results from the Pelvic Inflammatory Disease Evaluation and Clinical Health (PEACH) Randomized Trial. Am J Obstet Gynecol 2002;186:929-37.
39. Burstein GR, Gaydos CA, Diener-West M, Howell MR, Zenilman JM, Quinn TC. Incident *Chlamydia trachomatis* infections among inner-city adolescent females. JAMA 1998; 280:521-6.
40. LaMontagne DS, Fine DN, Marrazzo JM. *Chlamydia trachomatis* infection in asymptomatic men. Am J Prev Med 2003;24:36-42.
41. Turner CF, Rogers SM, Miller HG, et al. Untreated gonococcal and chlamydial infection in a probability sample of adults. JAMA 2002;287:726-33.
42. Howell MR, Gaydos JC, McKee KT Jr, Quinn TC, Gaydos CA. Control of Chlamydia trachomatis infection in female Army recruits: cost-effective screening and treatment in training cohorts to prevent pelvic inflammatory disease. Sex Transm Dis 1999;26:519-26.
43. Paavonen J, Puolakkainen M, Paukku M, Sintonen H. Cost-benefit analysis of first-void urine Chlamydia trachomatis screening program. Obstet Gynecol 1998;92:292-8.
44. Klausner JD, Chaw JK. Patient-delivered therapy for chlamydia: putting research into practice. Sex Transm Dis 2003;30:509-11.
45. Schillinger JA, Kissinger P, Calvet H, et al. Patient-delivered partner treatment with azithromycin to prevent repeated Chlamydia trachomatis infection among women: a randomized, controlled trial. Sex Transm Dis 2003;30:49-56.
46. Committee on Practice and Ambulatory Medicine. Recommendations for preventive pediatric health care. Pediatrics 2000;105: 645-6.
47. Screening for chlamydia and gonorrhea in adolescents. In: Health care for adolescents. Washington, D.C.: American College of Obstetricians and Gynecologists, 2003.
48. Hollblad-Fadiman K, Goldman SM. American College of Preventive Medicine practice policy statement: screening for *Chlamydia trachomatis*. Am J Prev Med 2003;24: 287-92.
49. Guidelines for adolescent preventive services. Chicago: American Medical Association, 1997. (Accessed November 19, 2003, at http://www.ama-assn.org/ama/upload/mm/39/gapsmono.pdf.)
50. Davies HD, Wang EE. Periodic health examination, 1996 update. 2. Screening for chlamydial infections. CMAJ 1996;154:1631-44.